REMARKS

Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 were pending in the application. Claims 1, 2, 4, 8, 10, 13, 15, 26, 27, and 28 have been amended. New claims 29-46 have been added. Claims 3, 11, 14, 16, 18, 19, and 21-25 have been cancelled. Accordingly, claims 1, 2, 4, 5, 7-10, 13, 15, and 26-46 are currently under examination.

Support for the amendments to claim 1 can be found in the specification at, for example, page 3, line 27. Independent claim 1 has been amended to more particularly recite the characteristics of the claimed transgenic animals. Support for the amendment to claim 1 can be found in the specification at least at page 31, lines 34-36. Certain claims have also been amended to correct dependencies. Support for the amendments to the claims and new claims 26-46 can be found throughout the specification and in the claims as previously pending. Additional support for the amendment to claim 13 and new claim 46 can be found in the specification at least at page 38, lines 2-4.

No new matter has been added by way of the amendments to the claims or the new claims. Applicants submit that the amendments herein will place the case either in condition for allowance or in better form for appeal. Accordingly, Applicants request that the amendments be entered. Amendment of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to more particularly point out and distinctly claim Applicants' invention in order to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Double Patenting Rejection

Claims 1-11 and 13-28 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-26 of U.S. Patent No. 5,866,755. Applicants thank the Examiner for acknowledging Applicants' request to hold the double-patenting rejection in abeyance until the claimed subject matter is indicated allowable.

Rejection of Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner states that while the specification is enabling with regard to a transgenic mouse, the specification "does not reasonably provide enablement for any other embodiment." The Examiner is of the opinion that Applicants have failed to enable non-human transgenic animals because "one can not predict the relationship between the gene of interest and the phenotype produced," and that the specification is not enabling for non-human transgenic animals in view of the unpredictability of the art. Applicants respectfully traverse this rejection.

The claims of the present invention are directed to a non-human transgenic animal having a transgene comprising a fusion protein comprising a first polypeptide which is a Tet repressor or mutated Tet repressor that binds to a *tet* operator sequence, operatively linked to a heterologous second polypeptide which inhibits transcription in eukaryotic cells integrated into the genome of the animal. In one embodiment, the non-human transgenic animal also has a *tet* operator-linked gene integrated into its genome. As amended, claim 1 specifies that the transgene is expressed in cells of the animal at a level sufficient to produce amounts of the fusion protein that are sufficient to inhibit transcription of the *tet* operator-linked gene, ***wherein the level of transcription of the tet operator-linked gene is less than the level of transcription prior to regulation by the fusion protein***. Amended claims 27 and 28 also specify that the *tet* operator-linked gene is expressed ***at detectable levels*** in cells of the transgenic non-human animal in the presence or absence of tetracycline or an analogue thereof.

Applicants provide working examples in the specification demonstrating the efficacy of the *tet* gene regulatory system, wherein transcription of a *tet* operator-linked gene of interest is controlled by a Tet repressor fusion protein. The specification provides at Example 6, a working example of a transgenic mouse which comprises the tTA regulatory system, wherein the gene of interest is the luciferase reporter gene. In Figure 12 and at pages 61-63 of the specification, Applicants demonstrate that transgenic mice carrying the tTA and *tet* operator-linked luciferase gene have decreased or increased luciferase activity based on the presence or absence of Tc. Based on these results,

Applicants show that the *tet* regulatory system like that described in the claimed transgenic non-human animal, is a ***predictable system*** which provides a precise mechanism for controlling expression of a gene of interest. Thus, in contrast to the Examiner's assertions, the claimed transgenic non-human animals of the present invention are predictable with respect to transgene behavior and transcriptional control, by virtue of the demonstrated reliability of the tTA regulatory system.

Further, in contrast to the Examiner's comments, ***the pending claims do not recite a phenotype limitation.*** The claimed invention is directed to a transgenic non-human animal comprising a Tet-based gene expression regulatory system, wherein transcription of a *tet*-operator linked gene of interest depends on the presence or absence of tetracycline or an analogue thereof. Transcription of the *tet*-operator linked gene is dependent upon the bound state of the inhibitor fusion protein on the *tet* operator. Thus, the "phenotype" resulting from the *tet* gene regulatory system is one of active or inactive gene expression. Gene expression of the *tet*-operator linked gene is higher in non-human transgenic animals with an unbound TetR inhibitory fusion protein than said animal with a bound TetR fusion protein, which inhibits transcription of the *tet*-operator linked gene.

The Examiner states that "the issue is not that one cannot produce a transgenic animal, but the issue is: if it is unpredictable to produce one with a phenotype, how can the animal be used." Applicants note that a predictable phenotype is not a required element of the pending claims, *i.e.*, the production of the transgenic animal as claimed is enabled. In addition, Applicants maintain that the scientific process calls for one to make an educated scientific estimation of the expected resulting phenotype which may or may not be correct upon completion of the experiment. If one of ordinary skill in the art could predict the phenotype of expression or lack of expression of a gene of interest, there would be no reason to create such a transgenic animal. Moreover, the ordinarily skilled artisan is able to make an educated estimate as to the phenotype which acts as his/her hypothesis in the scientific process.

The Examiner states that the references provided in Applicants' response of Dec. 8, 1999 are "directed to a certain gene of interest and a certain transgenic animal and these arts do not cure the unpredictability." The references provided in Applicants' previous response described examples of different cell types and transgenic non-human

animals other than mice which express various genes of interest controlled by the Tet gene regulatory system. The previous references were offered as a representative sampling of numerous *tet*-operator linked genes whose transcription has been controlled using the *tet* gene regulatory system of the invention. Applicants maintain that each reference is representative of a gene of interest and speaks to the predictability of transgenic expression using the claimed invention. Applicants have presented numerous examples of genes of interest under the control of the claimed *tet* gene regulatory system in different types of cells and animals, and are unsure, based upon the Examiner's comments, what additional information could possibly be required. Clarification is therefore requested.

In summary, Applicants submit that one of ordinary skill in the art could make and use the claimed invention based on general knowledge in the field of transgenic animals exemplified by the above-mentioned references and the teachings of the instant specification. Applicants provide working examples in the specification, as well as previously submitted references, which demonstrate gene transcription in transgenic non-human animals can be regulated by the claimed *tet* system. Applicants have shown that the *tet* gene regulatory system is a proven method of controlling gene expression. In addition, the enclosed references (see below) illustrate that not only were methods of making non-murine transgenic animals available at the time of filing of the priority application, but also that non-murine transgenic animals exhibit predictable phenotypes. Accordingly, Applicants respectfully request that the rejection of claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 under U.S.C. §112, first paragraph, be withdrawn.

Rejection of Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. In the Office Action of May 21, 2003, the Examiner states that "[t]he claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." In addition, the Examiner states,

"the phenotype(s) of the claimed animals can not be predicted because the art of making transgenic animals or knockout animals is highly unpredictable."

Applicants provide a transgenic non-human animal comprising a highly regulatable gene expression system, wherein a transgene encoding a fusion protein comprising a Tet repressor or a mutated Tet repressor is operatively linked to a transcriptional inhibitor and is integrated into the genome of the non-human transgenic animal. The fusion protein in turn inhibits or allows for the transcription of a gene which is operably linked to a *tet* operator through the presence or absence of tetracycline or a tetracycline analogue, thus providing a consistent and predictable regulatory system. As described above, ***Applicants note that the claimed invention specifies that expression of the tet-operator linked gene is less than the level of transcription prior to regulation by the fusion protein. The phenotype of the non-human transgenic animal is dependent on the gene of interest, and is not a required element of the claimed invention.***

As the Examiner is aware, possession of the invention may be shown in a number of ways. As specified in M.P.E.P. 2163, "[a] specification may describe an actual reduction to practice by showing that the inventor constructed an embodiment or performed a process that met all the limitations of the claims and determined that the invention would work for its intended purpose. (*Cooper v. Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed Cir. 1998)." As described above, Applicants describe working examples in the specification teaching transgenic mice comprising the *tet* gene regulatory system of the invention, wherein expression of the luciferase gene is controlled by a TetR activator fusion protein. Applicants teach that transcriptional inhibitor fusion proteins may also be used to control gene expression of a *tet*-operator linked gene, and provide working examples of how to construct such fusion proteins (see Examples 4 and 5). In addition, Applicants teach at pages 17-19 of the specification. that non-murine transgenic animals can be made comprising the *tet* gene regulatory system.

Applicants respectfully maintain that at the priority date of the instant application, the teachings set forth in the specification with regard to the general construction of transgenic organisms (see *e.g.*, pages 17-19), was routinely utilized in the production of a variety of transgenic organisms, including rats, rabbits, pigs, sheep, cows, and other

domestic farm animals. In addition to Applicants' previous comments regarding the cited Wood reference, Applicants refer the Examiner to the following references which are enclosed herewith, each of which supports the predictability of the presently claimed invention in animals other than mice.

Hammer *et al.* (1985) *Nature* 315: 680 (Appendix A – hereinafter "Hammer-1985") describes microinjection of a transgene construct encoding the mouse metallothionein -I (MT) promoter/regulator region fused to the human growth hormone gene (hGH) into pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. The authors analyzed the frequency of integration of the transgene in all three experimental species, as well as the expression of hGH in transgenic rabbits and pigs. The data described in Hammer-1985 show that the frequency of integration, equivalent to the number of transgenic offspring, was 12.8% in rabbits, 11% in pigs, and 1.3% in sheep (see Table I). Expression of the transgene revealed that out of sixteen rabbits, four had detectable levels of hGH specific mRNA in the liver. Thus, 25% of the successful transgenic animals exhibited detectable levels of the transgene. For transgenic pigs, eleven out of eighteen transgenic animals expressed the transgene, corresponding to 61%. Expression in sheep was not determined as only one transgenic sheep was obtained.

Mullins *et al.* (1990) *Nature* 344:541 (Appendix B – hereinafter "Mullins-1990") describes the introduction of the mouse *Ren-2* renin gene into rats and provides an analysis of the resulting phenotype, namely hypertension or elevated blood pressure. The results provided in Mullins-1990 demonstrate an integration frequency of 62.5%, as five out of eight rats carried the transgene. Four of the transgenic founder rats were bred successfully, and, notably, all four transgenic founder rats had elevated blood pressure compared to control animals that did not carry the transgene. Furthermore, analysis of a transgenic line established from a transgenic founder male rat demonstrated that, without exception, progeny inheriting the *Ren-2* renin transgene, also had the hypertensive phenotype.

The techniques described in Hammer-1985 and Mullins-1990 thus resulted in successful production of transgenic animals other than transgenic mice, including transgenic pigs, rabbits, and rats. Both Hammer-1985 and Mullins-1990 show that transgenic animals can be obtained through microinjection of DNA into the pronuclei or

nuclei of eggs as taught by the instant specification, which is the standard procedure still today, for generating transgenic animals. These references, therefore, demonstrate the efficacy of the techniques taught in the instant specification in the production of transgenic animals other than mice, and including pigs, rabbits, and rats. In addition, a recent paper, Auerbach *et al.* (2003) *Transgenic Research* 12:59 (enclosed herewith as Appendix C), shows that even today, transgenic mice are obtained at an overall frequency of 15-20% (see Table 2, page 63). Thus, the frequencies determined for rabbits (12.8%) and pigs (11%) in Hammer-1985, as well as the frequency obtained in the rats described in Mullins-1990 (62.5%), are well within the range of those observed for transgenic mice.

Applicants further submit herewith the first paper published on the use of the *tet* system in transgenic animals, namely Furth *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:9302 (Appendix D). While no numbers are given for the frequency of transgenic animals obtained, the results described in Furth demonstrate that six double transgenic mouse lines carrying both regulatory elements of the *tet* system, *i.e.*, a *tet* operator linked gene of interest and a TetR fusion protein comprising a transcriptional activator, were obtained which clearly show the expected phenotype, *i.e.*, β-galactosidase activity. To date, more than 150 mouse lines are available which carry the *tet* regulatory elements that allow precise control of a gene of interest using the *tet* gene regulatory system like that of the claimed invention. A summary of these lines is available in Berger and Bujard (2004) Novel Mouse Models in Biomedical Research: The Power of Dissecting pathways by Quantitative Control of Gene Activities. In: Handbook of Experimental Pharmacology, Vol. 159. Editors: S.Offermanns and L.Hein; Springer Verlag Berlin Heidelberg. Applicants therefore submit that at the priority date of the instant application (June 14, 1993), it was possible to obtain transgenic animals other than mice with a predictable phenotype as methods for generating and screening transgenic animals were well established in the field of transgenics.

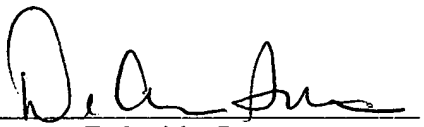
In view of the teachings in the specification, as well as the predictability of the art at the time of filing of the priority document, Applicants respectfully request that the Examiner withdraw the rejection of claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 under 35 U.S.C. § 112, first paragraph

SUMMARY

In view of the foregoing remarks, reconsideration of the rejections and allowance of all pending claims is respectfully requested.

If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicants' Attorney at (617) 227-7400.

Respectfully submitted,



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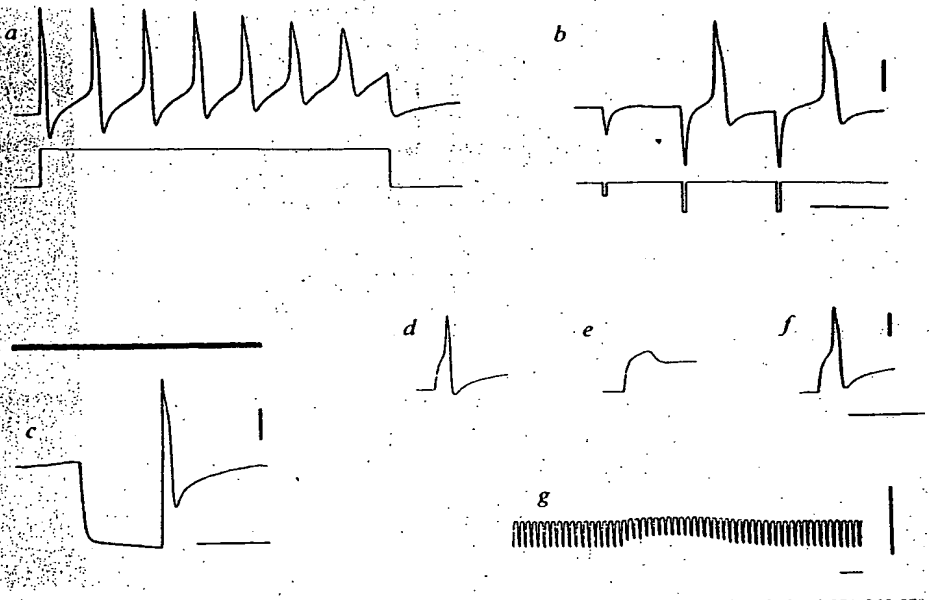

Fig. 2 Intracellular recordings from salivary gland cells of *H. ghilianii*. *a*, *b*, Action potentials, which may exceed 90 mV, are initiated by depolarizing current (*a*) and also following hyperpolarization by inward current (*b*; 100-ms pulses were applied). *c*, A hyperpolarizing current pulse in one salivary cell (lower trace) produces rebound excitation but there is no sign of electrical responses in an adjacent cell, recorded simultaneously at high gain on the upper trace. Experiments of this type indicate that the gland cells are not electrically coupled. *d–f*, Effect of 5 mM Co^{2+} added to the bathing solution. *d*, Control impulse in response to depolarizing current; *e*, 5 min after addition of $CoCl_2$ the action potential is abolished (a delayed rectification is apparent); *f*, recovery. This indicates that Ca^{2+} is the major current carrier for generation of action potentials. *g*, Brief application of 10^{-5} M serotonin produces a depolarization and increase in membrane conductance (indicated by reduction in amplitude of constant-current hyperpolarizing pulses). Voltage scales (vertical bars), 25 mV (1 mV in *c*, upper trace); time scales (horizontal bars), 2 s.

The natural stimulus for action potential generation, whether neural and/or hormonal, is unknown. Several putative neurotransmitters, however, (dopamine, serotonin and acetylcholine) were found to depolarize the gland cells, with an accompanying decrease in membrane resistance (Fig. 2*g*) and occasionally the production of no more than four impulses. Interestingly, in the presence of dopamine, applied depolarizing current was sometimes found to produce repetitive firing which was not simply a consequence of the depolarization produced by the drug. If the impulse provides a trigger for secretion, it seems unusual that the cells are normally so difficult to activate. Feeding, however, occurs very infrequently (every few months) and an action such as that of dopamine may mimic a natural process of bringing the gland into secreting condition.

In mammals, salivary and other exocrine gland cells are electrically inexcitable, producing graded potential changes (often hyperpolarizations) which may or may not be related to secretory function[10]. The *H. ghilianii* salivary cells are similar in their electrical excitability to mammalian endocrine cells such as those in the pancreas[12], adenohypophysis[13] and adrenal gland[14] (some molluscan exocrine glands produce action potentials[15]). This similarity extends to the anode-break excitation[8] shown by chromaffin[14] and anterior pituitary cells[13].

We have also found the *H. ghilianii* salivary gland to be suitable for molecular genetics because the cells have a very large ramifying nucleus that displays gene amplification of $\sim 10^6$ times; this should allow precise questions to be asked about the relationship between secretion and transcription/translation of identified genes. Thus, the *H. ghilianii* salivary gland, with its unusual combination of properties, represents a simple, accessible preparation with distinct experimental advantages for cellular studies of glandular secretion.

This specialized leech has been generally unavailable because in its natural habitat it is restricted to Amazonia. We have developed techniques for breeding this species and a facility has been set up by Biopharm to supply hementin to interested researchers.

Received 5 March; accepted 15 April 1985.

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Production of transgenic rabbits, sheep and pigs by microinjection

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Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin[1], *Candida elegans*[2], *Xenopus*[3], *Drosophila*[4,5] and mice[6–11]. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth[12,13]. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, *MT–hGH*[13], into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.

Studies with mouse ova indicated that integration of a gene into host chromosomes is much more efficient with nuclear than with cytoplasmic injection[14]. On this basis, we reasoned that nuclear injection would be an appropriate first approach with other species. The first problem encountered was visualization

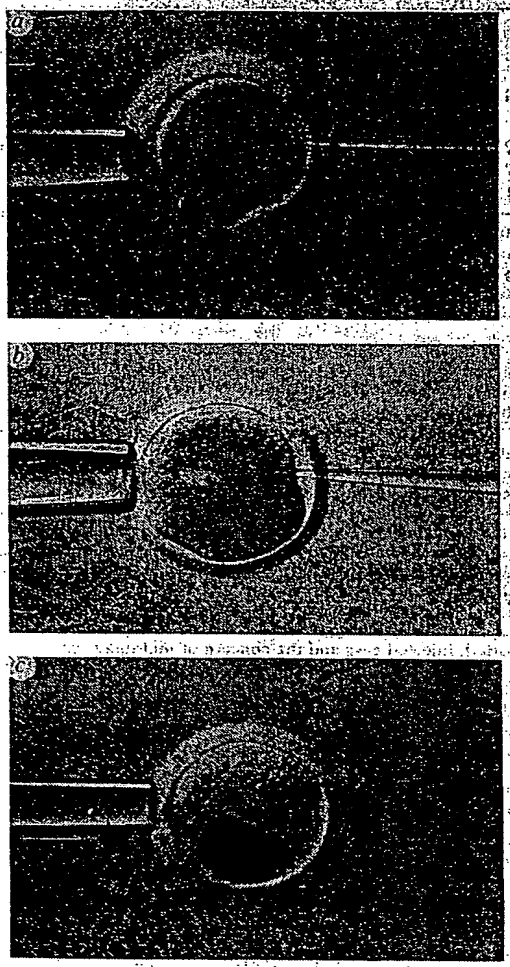

Fig. 1 Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (*a*), sheep (*b*) and pig (*c*) following microinjection. Ova are held by a blunt holding pipette (diameter ~50 μm); an injecting pipette (diameter ~1.5 μm) has penetrated the zona pellucida, plasma membrane and pronuclear envelope. The tip is seen within the nucleoplasm immediately following injection of buffer containing DNA. The porcine ova (*c*) has been centrifuged at 15,000*g* for 3 min to reveal the normally obscure pronuclei[15]. Visualization of nuclear structures is aided by the use of interference-contrast optics, and microinjection is carried out under ×250 magnification using a Leitz microinjecting apparatus[10,14]. Injection was monitored by observing the diameter of the pronuclus or nucleus, which was expanded ~50%.

of the pronuclei or nuclei in the ova. Rabbit nuclear structures are readily seen (Fig. 1*a*). However, pronuclei and nuclei in sheep ova are difficult to locate and can only be seen by fluorescent microscopy using DNA specific fluorochromes (Hoechst, 33258) or by interference contrast (IC) microscopy (Fig. 1*b*). The combination of stain and ultraviolet light is damaging to the ovum (data not shown), so we used IC microscopy for microinjection. Fluorescent analysis indicated that IC microscopy is an effective method for pronuclear localization in approximately 80% of fertilized sheep eggs. Pig ova are opaque and no nuclear structures can be seen even with IC microscopy, but we found that centrifugation of pig ova at 15,000*g* for 3 min stratifies the cytoplasm (Fig. 1*c*), leaving the pronuclei or nuclei visible[15].

Once the nuclei could be visualized, microinjection was performed as described previously[10,14]. A few hundred copies of a 2.6 kilobase (kb) linear fragment containing the *MT-hGH* gene (see Fig. 2) were injected. Approximately 5,000 ova were injected and subsequently transferred to foster animals; about 500 of these resulted in fetuses or neonates (Table 1). The frequency of *MT-GH* integration was similar in the rabbit (12.8%) and the pig (11.0%) and low in sheep (1.3%). These integration efficiencies are probably accurate for the techniques being used because they are based on a large number of animals. The reasons for the lower integration frequency in these species compared to the mouse where it is ~27% are unknown but could be related to factors such as the concentration of DNA, buffer composition, age of the ovum and the structure of the chromosomes[14].

The number of copies of the *MT-hGH* gene that integrated was estimated by quantitative dot hybridization. Figure 2*d* shows the quantitation method as applied to transgenic pigs. Gene copy numbers ranged from 1 to 490 copies per cell (Table 2). The DNA from some of the transgenic animals was also analysed by restriction enzyme digestion of the chromosomal DNA followed by agarose gel electrophoresis and Southern blotting. Figure 2*a* shows the results obtained when the DNA was restricted with *Eco*RI, an enzyme that cuts once within the injected DNA. The probe detects two prominent bands in several rabbits and pigs. One band is close to the length of the injected DNA fragment (2.6 kb) and probably represents a tandem, head-to-tail array of the *MT-hGH* genes as is typically observed in transgenic mice[10,12]. The other band is approximately twice that length and might represent a head-to-head dimer, but further analysis will be required to test that possibility. When the DNA was restricted with *Sst*I (Fig. 2*b*), an enzyme that cuts twice within the injected DNA, two bands of the expected size were observed in all of the pigs and rabbits. Analysis of the sheep sample with *Eco*RI (not shown) and *Sst*I (Fig. 2*c*) revealed bands that were inconsistent with an intact *MT-hGH* gene, suggesting that the DNA had been trimmed or rearranged prior to integration.

Expression of the integrated genes was examined by quantitating *MT-hGH* mRNA by solution hybridization (Table 2). Only 4 of 16 rabbits analysed had any detectable *MT-hGH* mRNA in the liver, but the level was substantial in one of these. In mice, the frequency of expression of this gene is close to 70% (ref. 13). In pigs, mRNA levels were measured only in tail or ear samples because we did not want to risk adverse consequences of liver biopsy. Although tail and ear tissues are not primary sites of *MT* gene expression, we detected low levels of *MT-hGH* mRNA in several of the transgenic pigs (Table 2).

Plasma samples taken from pigs at birth and ~1 month later were analysed for hGH by radioimmunoassay. At birth, 11 of 18 pigs had detectable levels of hGH, ranging between 2 and 730 ng ml^{-1} (Table 2). One month later, hGH exceeded 300 ng ml^{-1} in three pigs. One rabbit also had a high level of hGH. Serum hGH as high as 64,000 ng ml^{-1} has been detected in transgenic mice, but accelerated growth rate was observed at levels of 20 to 80 ng ml^{-1} (ref. 13). None of these animals were exposed to high levels of zinc, a treatment that has been shown to activate *MT-hGH* gene expression ~10-fold in mice[13].

The effects of hGH on the growth of rabbits cannot be evaluated at present because only one live rabbit had detectable serum hGH and unfortunately it had malocclusion that impaired normal food consumption. Early indications are that the levels of hGH found in these transgenic pigs do not increase body weight dramatically. This may not be surprising considering that daily injections of bacterially synthesized hGH had no effect[16], and exogenous, highly purified porcine GH only stimulated growth by 10% when delivered during the major growth phase of the pig[17]. Transgenic offspring and littermate controls will need to be raised on normal and zinc-supplemented diets to determine precisely the effects of hGH on growth rate and other nutritional as well as endocrine parameters.

These experiments demonstrate that foreign genes can be introduced into several large animal species by microinjection of ova. Furthermore, expression of MT-hGH was obtained in rabbits and pigs. We used a fusion gene that has worked well in mice to demonstrate the feasibility of such techniques, and we are now trying several modifications in an effort to improve the level of expression and physiological response.

Table 1 Efficiency of producing *MT-hGH* transgenic rabbits, sheep and pigs by microinjection

Species	Transferred injected ova	Recipients	Integration frequency (%)	Expression frequency *MT-hGH* mRNA	Expression frequency Serum or plasma hGH
Rabbit*	1,907	73	28/218 (12.8)	4/16	1/1
Sheep†	1,032	192	1/73 (1.3)	ND	ND
Pig‡	2,035	64	20/192 (10.4)	11/20	11/18

Integration frequency is the number of animals (fetuses, stillborns and neonates) that retained the injected DNA/total number of animals resulting from injected ova. Six gilts bearing only injected eggs farrowed, producing 52 neonates, 5 of which retained DNA. In 31 gilts that farrowed, 204 fertilized control ova were transferred along with 859 injected ova to ensure sufficient embryos at implantation to maintain pregnancy. If survival of injected eggs to fetuses (16.4%) was similar for both groups, then injected eggs resulted in 140 of 252 fetuses and piglets produced, 15 of which retained injected DNA. We combined the data from the two groups to estimate integration efficiency. Expression frequency is the number of fetuses or neonates containing *MT-hGH* mRNA or plasma hGH per total number of animals examined. ND, not determined.

* Fertilized one-cell rabbit ova were flushed from the oviducts of superovulated New Zealand White (NZW) females 19 h after mating[20]. For microinjection the ova were placed in the well of a depression slide containing ~100 μl modified BMOC culture medium[21] with the $NaHCO_3$ replaced by 25 mM HEPES[22] and covered by silicone oil. Microinjection of embryos (1,857 one-cell and 50 two-cell) was performed as described for mouse ova[10,14]. Following injection, the ova were washed in fresh modified BMOC and surgically transferred to the oviducts of synchronized pseudopregnant rabbits[23].

† Rambouillet ewes were superovulated after exhibiting at least one prior oestrus period. On about day 10 of the oestrous cycle, progestagen-impregnated vaginal sponges (6α-methyl-17α-acetoxy progesterone, 60 mg; from Dr J. Lauderdale, Upjohn) were inserted and left for 12 days. Gonadotropin treatment (porcine follicle stimulating hormone, Burns) began three days before sponge removal and was continued twice daily (2.5 mg per injection, intramuscular) until the day following sponge removal[24]. At the onset of oestrus, ewes were either hand mated to fertile rams or inseminated *in utero* with 0.2 ml per horn of washed ram semen; 72 h after sponge removal, one-cell fertilized ova and cleaved ova were surgically collected from the reproductive tracts of anaesthetized ewes by flushing 6 ml Ham's F-10 medium containing 10% heat-inactivated fetal calf serum (FCS) from the utero-tubal junction through the cannulated infundibular end of each oviduct. The flushings were collected in sterile Petri dishes, and ova were removed under a dissecting scope. Ova were transferred to fresh Ham's F-10 containing 10% FCS and transported (~2.5 h) to Philadelphia in temperature-controlled containers. Microinjection of embryos (641 one-cell, 375 two-cell and 16 four-cell) was performed as previously described[10,14]. After embryos were injected, they were washed and transported to Beltsville. Embryos were aspirated into a glass micropipet tip with 10 μl Ham's F-10 and expelled 1-3 cm into the fimbriated end of the oviduct in synchronized recipient ewes. To assess the effects of transport and microinjection of DNA on egg development, a number of recipients bearing control and injected eggs were flushed 8 days following transfer. In recipients in which eggs were recovered, 26% of transported, uninjected and 10% of injected sheep eggs developed to blastocysts. Because of the high mortality of transported, injected eggs and the concern of multiple births, 5 or 6 embryos were transferred per recipient.

‡ Mature gilts were superovulated and bred as previously described[15]. At 18 to 27 h after the expected time of ovulation, gilts were anaesthetized and one cell fertilized the oviduct with 20 ml modified BMOC[21]. Ova were transferred to fresh BMOC and transported to Philadelphia. Microinjection of embryos (316 one-cell, and 1,719 two-cell) was performed as previously described[10,14]. The obscured pronuclei or nuclei or one- and two-cell pig ova were visible after centrifugation for 3 min at 15,000 *g*. Centrifugation of pig ova at this force and length of time has no detectable effect on development[15]. After embryos were injected, they were transported to Beltsville and transferred to the oviducts of recipient gilts as previously described[15]. To assess the effects of transport and microinjection of DNA on egg development, recipients bearing control and injected eggs were flushed 5 days following transfer. Approximately 52% of transported, uninjected and 23% of injected pig eggs developed to blastocysts. The pregnancy rate in recipients bearing only injected eggs was 50% while in recipients bearing both injected and control eggs 58% farrowed.

Table 2 Characteristics of transgenic rabbits, sheep and pigs

Species	Animal and sex	Gene copy (no. per cell)	*hGH* mRNA (molecules cell^{-1})	Immunoassayable hGH (ng ml^{-1})
Rabbit	59-3*	20	0	
	64-2♀	18	0	
	68-3†	28	39	ND
	68-4♀	24	0	
	122-9♀	11	0	
	131-8†	88	0	
	157-1♀	3	0	
	163-3♂	3	15	250
	167-5♀	10	0	
	179-1♀	16	0	
	179-2♂	36	0	
	179-5♀	6	0	
	200-3*	8	920	ND
	221-1*	40	140	ND
	223-4*	5	0	
	223-5*	40	0	
Sheep	693-1♀	1	ND	ND

Species	Animal and sex	Gene copy (no. per cell)	*hGH* mRNA (molecules cell^{-1})	Immunoassayable hGH (ng ml^{-1})
Pig	100-3*	4	0	ND
	163-4*	140	0	ND
	3-2♂	330	26	Neg.
	3-6♂	490	53	17
	7-3♀	90	12	80
	10-4♀	23	0	Neg.
	11-2♂	1	0	40
	16-3♀	3	0	Neg.
	16-8♀	10	18	53
	16-9♂	1	5	65
	17-4♀	3	0	Neg.
	18-3♀	3	6	60
	20-2♂	2	4	40
	20-8♂	110	1	2
	21-4♂	1	2	Neg.
	21-5♀	1	0	Neg.
	22-1♀	50	24	56
	23-8♀	7	41	730
	25-2♀	17	0	108
	25-4♀	2	0	Neg

A 2.6-kb linear fragment of the fusion gene *MT-hGH*[13] containing the mouse MT-I regulator/promoter fused to hGH[19] was injected into fertilized one-cell and two-cell rabbit, sheep and pig eggs as described for mouse ova[10,14]. The male or female pronuclei of one-cell ova and both nuclei of two-cell ova were microinjected with a 3 ng μl^{-1} solution of DNA in Tris-EDTA buffer[14]. The ova were transferred into the oviducts of recipients at the same stage post oestrus as the donors (see Table 1). Animals without identified gender were either killed as fetuses(*) or were stillborn(†). The number of foreign fusion genes per cell was estimated by extracting total nucleic acids from a piece of fetal liver, neonatal ear or tail samples and performing quantitative dot hybridization with a 1.0-kb *Pou*II probe spanning most of the *hGH* structural gene[19] (see Fig. 2). *MT-hGH* mRNA was measured by solution hybridization with a ^{32}P-labelled oligonucleotide (21-mer)[25]. For rabbits, either a partial hepatectomy was performed or fetal liver was used. For pigs, the *MT-hGH* mRNA content of ear or tail samples was quantitated. The concentration of *hGH* was measured in pig plasma obtained shortly after birth and serum from a 9-month-old rabbit. Samples were assayed in duplicate at 2.5 and 10 μl by radioimmunoassay using a hGH kit provided by Dr Raiti (National Hormone and Pituitary Program). The assay did not cross-react with porcine GH but required extra normal rabbit serum and anti-rabbit gammaglobulin to quantify hGH in rabbit samples. Pigs with hGH values less than 2 ng ml^{-1} at birth were designated negative for hGH. At about one month of age, these pigs were also negative for hGH. ND, not determined; Neg, negative for hGH.

The key element in our success was the ability to visualize pronuclei and nuclei. Microinjection of the *MT-hGH* gene into the cytoplasm of 485 pig ova failed to produce DNA integration in 42 fetuses. Separate techniques for sheep and pigs were necessary because the opacity of the eggs differed. Although both contain dense cytoplasm, centrifugation did not help visualize pronuclei of sheep eggs and IC microscopy did not allow nuclear localization in pig ova. Preliminary work indicates

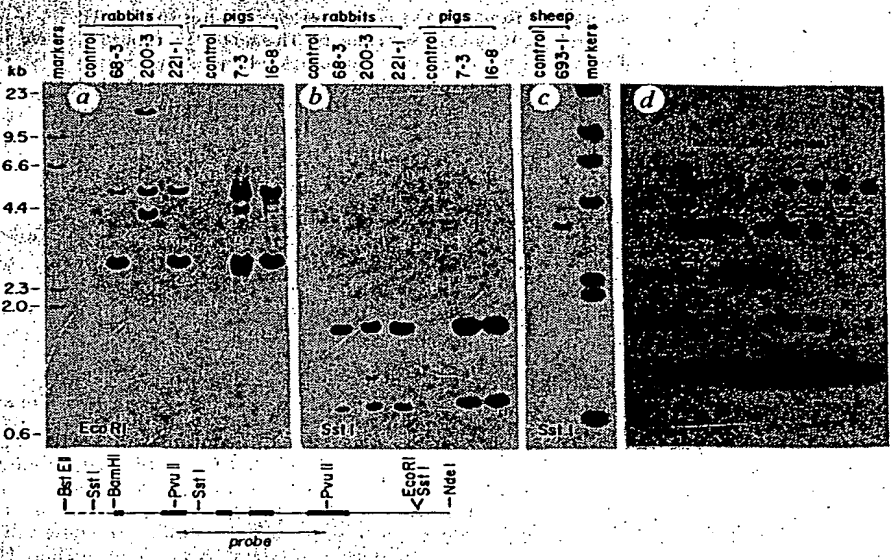

Fig. 2 Analysis of *MT-hGH* DNA introduced into rabbits, pigs and sheep. The diagram at the bottom shows the 2.6-kb *Bst*EII/*Eco*RI DNA fragment isolated from *MT-hGH* gene plasmid 111 that was microinjected[13]; the mouse MT-I promoter region is dashed, the *hGH* gene is solid, with the exons indicated as boxes, and the residual pBR322 sequences are dotted. The internal *Pvu*II fragment was isolated, nick-translated and used as a probe for quantitation of genes and Southern blots. Panels *a*, *b* and *c*: DNA (5 μg for controls and transgenic animals 200-3, 16-8 and 693-1; 1 μg mixed with 4 μg of control DNA for 68-3, 221-1 and 7-3) were digested with the indicated restriction enzymes (10 units; 6 h), electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated probe, washed and autoradiographed as described previously[18]. *a* and *b*, Exposure was 4.5 h; *c*, 24 h. For quantitation of gene copy number, 5 μg of DNA was spotted in duplicate onto nitrocellulose along with standards of 0, 0.5, 1, 2 and 5 μg of human DNA mixed with control DNA to make a total of 5 μg. *d* Shows the visualization *MT-hGH* gene copy number in transgenic pigs. After exposure, the spots were cut out and the radioactivity determined in a scintillation counter. Gene copy numbers were calculated from the standards assuming that the genome size of pigs and humans are comparable and that diploid human cells contain 10 genes homologous to the *Pvu*II fragment used as probe[19]. The results are shown in Table 2.

that these two techniques can be used for ova of other species; for example, IC microscopy allowed visualization of pronuclei in goat ova (unpublished observations) and centrifugation allowed localization of pronuclei in cow ova[15]. Although improvements in integration efficiency should be possible, the techniques have immediate application for both scientific and practical purposes.

We thank Mary Chandlee, Dennis McDuffie, Anne Powell, Leah Schulman, Myrna Trumbauer and Mary Yagle for technical assistance and Kenneth Bender, Paul Graninger, James Piatt, David Sherman and Stephanie Mengel for animal care. This work was supported in part by grants from the USDA (Section 1433 formula funds) and NIH (HD-19018) to R.L.B. and NIH (HD-09172) to R.D.P. R.E.H. was supported by an NIH training grant (HD-07155) and K.M.E. by an NIH postdoctoral fellowship (HD-06210).

Received 8 February; accepted 19 April 1985.

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Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells

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Haemophilia B, or Christmas disease, is an inherited X-chromosome-linked bleeding disorder caused by a defect in clotting factor IX and occurs in about 1 in 30,000 males in the United Kingdom[1]. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, because of impurities in the factor IX concentrate presently in use, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis and the virus causing acquired immune deficiency syndrome (AIDS)[2]. Because of the recent concern about the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable. Here, we report that after introduction of human factor IX DNA clones[3] into a rat hepatoma cell line using recombinant DNA methods, we were able to isolate small amounts of biologically active human factor IX.

Factor IX is a plasma glycoprotein which has an essential role in the middle phase of the intrinsic clotting pathway[4] where, in an activated form, IXa, it interacts with factor VIII, phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where it undergoes three distinct types of post-translational modification before secretion into the bloodstream as a 415-amino-acid-long, highly modified protein. These modifications are the vitamin K-dependent γ-carboxylation of 12 glutamic acid residues[5], the addition of several carbohydrate residues[6] and the β-hydroxylation of a single aspartic acid residue[7]. The first two modifications are known to be required for activity of factor IX[5,6]. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX, derived from factor IX DNA clones, would be most likely to succeed in a hepatic cell or a transformed cell line derived from a hepatocyte. None of the standard mammalian hepatoma cell

families (solid line) and acute families (dotted line). The peak multipoint lod score for chronic SMA is 9.03, and the peak lod score for acute SMA is 2.02. Pairwise lod scores for chronic and acute SMA families versus four markers located in the middle of the linkage region are shown in Table 1. The maximum two-point lod score for chronic families is 8.43 at a recombination fraction of 2% with marker D5S6, and 1.71 for acute families at a recombination fraction of 2% with marker D5S78.

Application of the HOMOG program[13] to the multipoint lod scores of the families with chronic SMA gave no evidence for heterogeneity among these families. Although the power of homogeneity tests can be lower in recessive families than in larger families with dominant diseases, the absence of evidence for heterogeneity led us to adopt the most parsimonious solution of assuming homogeneity. The confidence interval for the location of the gene for chronic SMA is 11 centimorgans (cM) wide and spans a region 2 cM proximal of locus D5S6 to a point 4 cM proximal of locus D5S78 (note arrows in Fig. 1). For families with acute SMA, the maximum lod score of 2.02 indicates that a gene responsible for this disease maps to the same general area. The best estimate for the location of the acute SMA locus is 15 cM distal to the estimated position of the locus for chronic SMA.

Our data indicate that clinically heterogeneous forms of chronic childhood SMA (type II or intermediate form and type III or Kugelberg–Welander or mild form) map to a single locus on chromosome 5q. The chronic forms of childhood-onset SMA, therefore, are likely to occur as the result of allelic heterogeneity, similar to the case for Duchenne- and Becker-type dystrophies[15]. It is interesting that our data indicate that acute childhood SMA (type I or Werdnig-Hoffmann or infantile SMA or severe SMA) map to the same, or a closely linked, locus on 5q. Other informative acute families must be analysed to confirm the linkage of this form of SMA and to evaluate the associated map location relative to that of chronic SMA. Also, other chronic families must be analysed to further assess the possible occurrence of nonallelic heterogeneity. It will be interesting to determine whether adult-onset and dominantly inherited cases of SMA similarly map to chromosome 5q. The gene encoding hexosaminidase B maps between markers D5S39 and D5S78 (refs 16, 17). Deficiencies in both the α- and β-subunit of this enzyme have been associated with chronic cases of SMA[18,19]. We are investigating whether this gene is a candidate for an SMA mutation. □

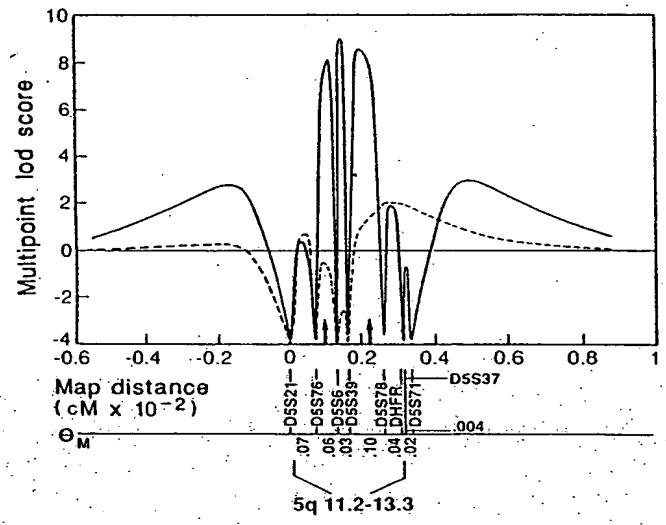

FIG. 1 Multipoint linkage analysis of the SMA disease locus with eight DNA markers spanning ~30 cM, including 5q11.2–5q13.3 (refs 16, 21). Analysis of seven chronic families (solid line) and six acute families (dotted line). Three chronic families each consists of four affected children and 8–12 unaffected sibs. Four chronic families each have three affected children and 0–4 unaffected sibs. The acute families, collected over a 3-year period, include one family with three affected children (trizygotic triplets), four families with two affected children, and one family with one affected and two unaffected sibs. Recombination fractions (θ_M) between DNA markers were calculated from published map distances[16]. Marker loci D5S6, D5S39, D5S78 and DHFR map to 5q11.2–13.3 (ref. 21). For the female-to-male distance ratio we used the published value of 1.6 as being appropriate for this area of the genome[22]. Multipoint lod scores were obtained by five-point analysis in all families, except one for which, for reasons of computational efficiency, three-point lod scores had to be calculated. The computer program used was LINKMAP of the LINKAGE package[20]. The confidence interval for chronic families (defined as points on the map with lod scores $\geq Z_{max}^{-1}$ where Z_{max} is the value of $Z(\theta)$ at the maximum likelihood estimate of θ) spans an 11-cM region marked by arrows at map positions 0.11 and 0.22.

Received 11 January; accepted 16 February 1990.

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ACKNOWLEDGEMENTS. We are indebted to the SMA families whose cooperation and support made this project possible, and also to the early contributions of Dr Michael Mendelsohn. Thanks to Barbara Byth, Dr Sally Candy, and Bartolome Jaume-Roig for technical assistance, Linda Skerry for family coordination, Dr Kamma Das for tissue culture, and Dr Lewis P. Rowland for helpful discussions. This work was supported by the Muscular Dystrophy Association of America, the Muscular Dystrophy Group of Great Britain and Northern Ireland, the MRC of Great Britain, and the W. M. Keck Foundation.

Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene

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PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized[1–8], including those of the renin–angiotensin system, which plays a central part in blood pressure control[9–10]. Here we describe the introduction of the mouse *Ren-2* renin gene[3,11–13] into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

We chose the mouse *Ren-2* renin gene for introduction into the rat germline because it had already been characterized in transgenic mice and because we expected it to be highly

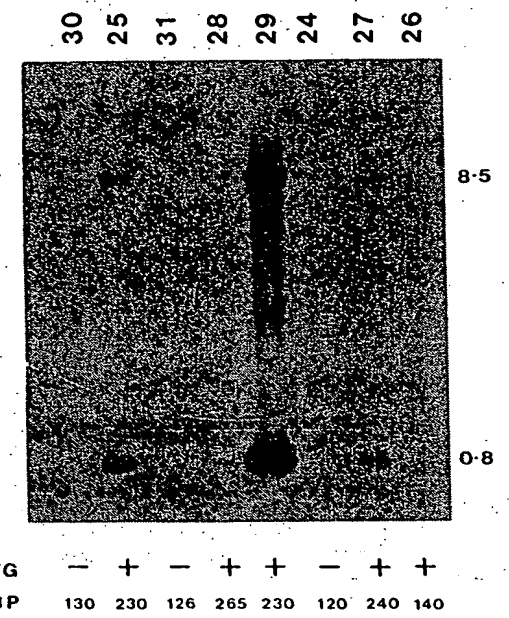

FIG. 1 Southern blot identifying animals carrying the DBA/2 *Ren-2* gene. The identification numbers of potential founder animals are shown above the corresponding lane and the positions of the *Ren-2*-specific 8.5-kb and 0.8-kb restriction fragments are indicated to the right. Transgenic (TG) positive and negative animals are indicated by symbols under the corresponding lane, together with the systolic blood pressure (BP, in mm Hg) of each animal at the age of 10 weeks.
METHODS. DNA preparation: DNA was prepared from tail biopsies and digested with *Pvu*II. After electrophoresis on a 0.8% agarose gel, samples were Southern-blotted and hydridized with a ^{32}P-labelled dCTP 300-bp probe derived from the renin complementary DNA clone pDD1D2[17] and labelled by random priming[18]. Preparation of transgenic animals: DNA was prepared for microinjection by digestion of the cosmid clone cosDBA-1 (ref. 17) with *Xho*I, and subsequent isolation of the 24-kb *Xho*I fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol-precipitation before being centrifuged on a CsCl gradient[19]. DNA was diluted to a final concentration of 1 μg ml^{-1} in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at −20 °C before use. Fertilized eggs were derived from a cross between Sprague-Dawley female and WKY male rats after superovulation of immature females (at 4 weeks old) according to the procedure of Armstrong *et al.*[20]. Eggs were cultured, microinjected, and re-implanted as described for the mouse[19]. Rats were all bred in our own facilities.

expressed in certain tissues[14]; also, injection of purified mouse submandibular gland (SMG) renin (encoded by *Ren-2*) into rats leads to a significant and sustained increase in blood pressure[15]. Fertilized rat eggs were microinjected with a linear DNA fragment containing the entire DBA/2J *Ren-2* gene, including 5.3 and 9.5 kilobases (kb) of 5′ and 3′ flanking sequence, respectively[14]. From 37 eggs implanted, there were eight progeny, of which five carried the transgene (Fig. 1). Four of the founders were bred successfully and three of them (TGRmRen2, numbers 25, 26 and 27) transmitted the transgene to their progeny. At ten weeks of age and before breeding, the blood pressure of the founder animals was measured. For four of the transgenic animals it was in the range 230–265 mm Hg, but was 120–130 mm Hg in the transgene-negative litter-mates (Fig. 1). Breeding of TGRmRen2 female 26, who was not hypertensive, revealed her to be mosaic for a transgene insertion site, the inheritance of which segregated with hypertension in the blood pressure range indicated (data not shown). The phenotype is therefore independent of the transgene insertion site and is not due to a fortuitous mutation associated with the integration event.

Analysis of the transgenic line established from TGRmRen2 male 27 revealed that, without exception, progeny inheriting the transgene also had the hypertensive phenotype. Both male and female animals of this line developed hypertension rapidly, beginning at four weeks of age and reaching a maximum by nine weeks (Fig. 2*a*). Pharmacological intervention to reduce blood pressure took the form of treating the animals with 10 mg kg^{-1} per day of the converting enzyme inhibitor, captopril; this inhibits the conversion of angiotensin I to angiotensin II. This low dose, given daily in the drinking water, was sufficient to reduce the blood pressure of the hypertensive transgenic rats reproducibly by 40–60 mm Hg (Fig. 2*b*), indicating that the hypertension is largely dependent on the conversion of angiotensin I to angiotensin II.

Northern blot analysis showed that the concentration of renin transcripts was high in the adrenal glands of the transgenic animals (Fig. 3*a*). In addition, renin transcripts were detectable in testis, coagulation gland, thymus and small intestine in transgene-positive animals, but not in control transgene-negative littermates (data not shown). These additional sites represent tissues in which renin is naturally expressed in the mouse. Renin messenger RNA was not observed in the SMG, a result that could reflect the absence of essential *trans*-acting factors in this tissue as the endogenous rat renin gene is not expressed in the SMG (ref. 16). An RNase protection assay using a *Ren-2*-specific probe confirmed that the renin transcripts in the adrenal gland were exclusively of *Ren-2* origin and that *Ren-2* transcripts were present in the kidneys of transgene-positive animals (Fig. 3*b*).

No evidence was found for altered plasma angiotensinogen levels, but plasma renin activity and angiotensin I were significantly lower in transgenic animals than in the controls (Fig. 4*b–e*). The amount of angiotensin II was also less than in the

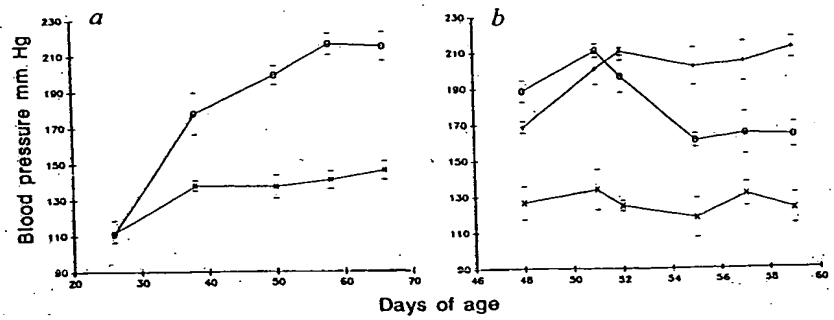

FIG. 2 *a*, Development of blood pressure with age. Each point represents the mean of 7 (transgenic, circles) or 5 (control, crosses) animals and standard errors are indicated above and below each data point. *b*, Effect of converting enzyme inhibitor (CEI) on blood pressure. Each point represents the mean of 3 animals and standard errors are indicated above and below each data point. +, TGRmRen2 L27 rats having no treatment; O, TGRmRen2 L27 rats receiving CEI; ×, control rats receiving CEI.
METHODS. Blood pressure was determined by tail plethysmography under light ether anaesthesia as described[21]. Animals under converting enzyme inhibitor treatment were given captopril (10 mg kg^{-1} per day) in their drinking water. Captopril treatment started at day 51.

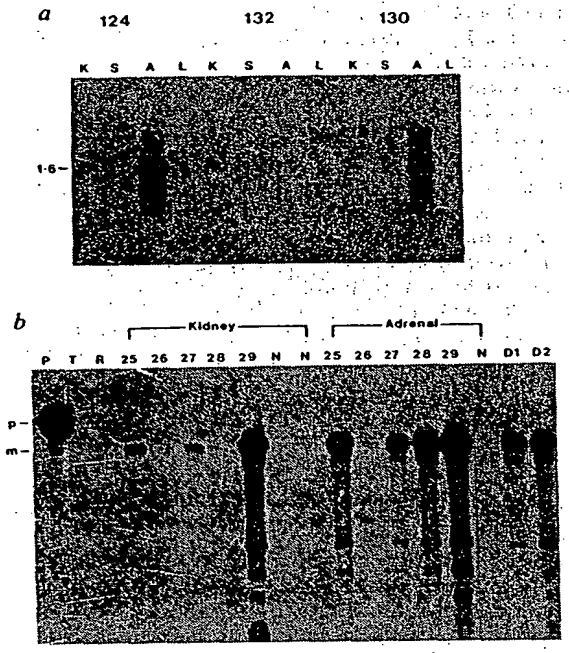

FIG. 3 Northern blot and RNase protection assay. *a*, Northern blot of RNA isolated from the kidney (K); SMG (S); adrenal gland (A); and liver (L) of transgene-positive (124 and 130) and transgene-negative (132) male rats. The size of the hybridizing RNA is indicated in kb. With the exception of the adrenal gland (5 μg), 40 μg total RNA was used for each sample. *b*, RNase protection of kidney and adrenal gland RNA from transgenic rats (numbers 25–29) and control littermates (N). The following controls are included: P, undigested probe; T, transfer RNA (9 μg); R, rat kidney RNA (20 μg); D1 and D2, mouse (DBA/2J) kidney RNA (20 μg and 40 μg, respectively). The positions of the undigested 244-nucleotide probe (p) and the 224-nucleotide mouse-specific protected fragment (m) are indicated.
METHODS. Preparation of RNA: Total RNA was isolated from mature rats as previously described[3] or by homogenization in guanidine isothiocyanate.[22] Northern blot analysis: Northern blots were prepared and hybridized as previously described[23] with a ^{32}P-labelled renin cDNA probe (pDD1D2) by random priming[18] and washed with 0.1 × SSC, 0.1% SDS at 65 °C. RNase protection assay: ^{32}P-labelled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM (ref. 15) using SP6 RNA polymerase. This transcript comprised 224 nucleotides of *Ren-2* antisense RNA and 20 nucleotides of vector-encoded sequence. Samples were dissolved in 30 μl 80% formamide, containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA and 200,000 c.p.m. of the gel-purified transcript, denatured at 100 °C for 1 min and incubated at 45 °C for 20 h. RNase digestion was performed in 300 μl buffer containing 40 μg ml^{-1} RNase A (Sigma) and 2 μg ml^{-1} RNase T1 (Calbiochem) for 45 min at 37 °C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels.

controls but the difference was not statistically significant. Determination of prorenin showed it to be raised in the plasma of transgenic animals (Fig. 4*a*), but the functional significance of this finding is unclear. Adrenal glands of the transgenic animals contained significantly increased renin concentrations (Fig. 4*f*). No evidence was found for the storage of renin in this tissue, so the large difference between renin mRNA levels and enzyme activity may reflect a constitutive secretion of *Ren-2*-derived renin from the adrenal glands. By contrast, kidney tissue from transgenic animals contained only 20–25% of the renin activity of the controls, which is consistent with immunocytochemical and ultrastructural data showing a reduction in renin storage granules in the juxtaglomerular apparatus (S. Bachmann *et al.*, manuscript in preparation) and suggests that renin expression is subject to translational or post-translational control. Preliminary studies on isolated kidney show that renin secretion is reduced and that there are no other abnormalities of renal function (K. Munter, personal communication).

Although we have defined a genetic basis for this transgenic hypertensive rat model, the mechanism responsible for elevating blood pressure remains to be established. The hypertension is clearly not due to overexpression of renin in the kidney, and the suppression of active renin in the kidney and in the plasma is probably a result of an already elevated blood pressure in young animals, pressure-mediated renin suppression being a well known phenomenon. The increased plasma prorenin probably originates, at least in part, from the adrenal gland, but the ovary, vascular tissue and other sources of prorenin should also be considered. Any role of prorenin in hypertension still awaits investigation, but in this respect it is interesting that prorenin is raised and still persists after nephrectomy in hypertensive patients, confirming that its origin is extra-renal. At this stage, the most likely explanation for the high blood pressure in TGRmRen2 rats is a stimulated renin–angiotensin system in the adrenal gland, with the consequent overproduction of steroid hormones. This is in keeping with our preliminary data on

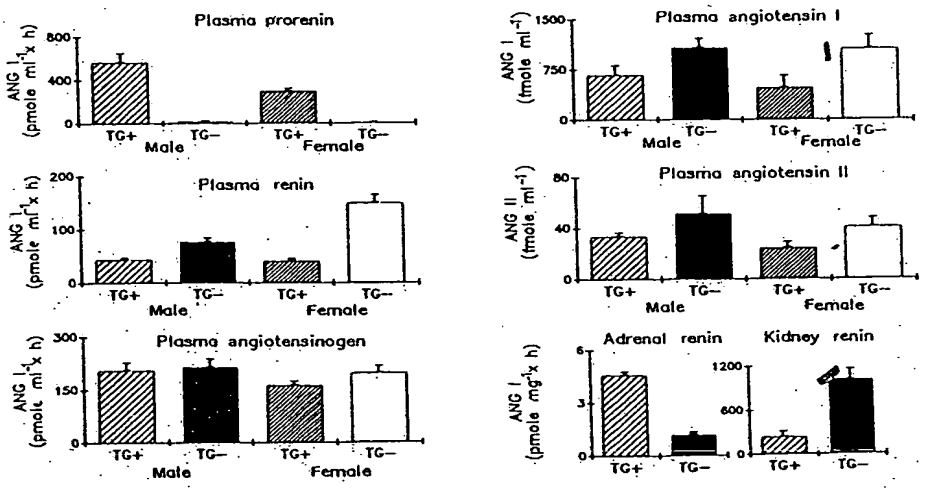

FIG. 4 Determination of plasma and tissue renin-angiotensin system components. Values represent the mean and standard error of 7 animals for each determination, with the exception of the kidney and adrenal gland renin values (3 animals). Statistical analysis by ANOVA showed the following significance values: prorenin, $P < 0.05$ between the transgenic animals and the corresponding controls; renin, $P < 0.005$ between the transgenic animals and the corresponding controls; angiotensin I, $P < 0.05$ between the transgenic animals and the corresponding controls; tissue renin, $P < 0.01$ for the adrenal gland and $P < 0.005$ for the kidney.
METHODS. Concentrations of angiotensinogen, angiotensin I, angiotensin II and renin were determined as described[24,25]. Prorenin levels were calculated by subtraction of renin activity from total plasma renin activity determined after trypsin activation[26].

elevated urinary aldosterone excretion in male TGRmRen2 rats (15.4 ± 2.26 ng per 24 h) compared with controls (8.97 ± 1.06 ng per 24 h). These animals will enable us to study normal or low plasma renin hypertension and have shown us that renin can participate in the genesis of hypertension in a more subtle way than previously supposed. The construction of transgenic rats will therefore provide new opportunities for research into cardiovascular mechanisms. □

Received 14 November 1989; accepted 9 February 1990.

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ACKNOWLEDGEMENTS. We thank Dr K. W. Gross for pCOS-1 and pSLM, Dr D. Armstrong for advice and communication of unpublished results, Frank Zimmerman and Gesa Wernicke for technical assistance, Dr L. J. Mullins for critically reading the manuscript, and Drs U. Ganten, Yi Zhao and M. Lee for their contributions. This work was supported in part by the Deutsche Forschungsgemeinschaft, Squibb/von Heyden Co., Munich and The Commission of the European Communities, Concerted Action Program TRANSGENEUR.

Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses

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IN mammalian muscle, the subunit composition of the nicotinic acetylcholine receptor (AChR) and the distribution of AChRs along the fibre are developmentally regulated. In fetal muscle, AChRs are distributed over the entire fibre length whereas in adult fibres they are concentrated at the end-plate[1]. We have used *in situ* hybridization techniques to measure the development of the synaptic localization of the messenger RNAs (mRNAs) encoding the α-subunit and the ε-subunit of the rat muscle AChR. The α-subunit is present in both fetal and adult muscle, whereas the ε-subunit appears postnatally and specifies the mature AChR subtype[2–4]. The synaptic localization of α-subunit mRNA in adult fibres may arise from the selective down-regulation of constitutively expressed mRNA from extrasynaptic fibre segments. In contrast, ε-subunit mRNA appears locally at the site of neuromuscular contact and its accumulation at the end-plate is not dependent on the continued presence of the nerve terminal very early during synapse formation. This suggests that ε-subunit mRNA expression is induced locally via a signal which is restricted to the end-plate region and is dependent on the presence of the nerve only during a short period of early neuromuscular contact. Evidently, several mechanisms operate to confine AChR mRNAs to the adult end-plate region, and the levels of α-subunit and ε-subunit mRNAs depend on these mechanisms to differing degrees.

Hybridization of longitudinal sections of adult rat soleus muscle with ε- and α-subunit-specific antisense complementary RNA (cRNA) probes revealed strong hybridization signals at sites that had been previously identified as end-plates by staining for acetylcholinesterase (AChE). Figure 1*a* shows the end-plate region of a muscle stained for AChE. Subsequent hybridization with the ε-subunit-specific antisense probe showed a strong signal at the site where the AChE stain had been (Fig. 1*b*). After a brief exposure, groups of grains could be resolved above individual synaptic nuclei (Fig. 1*c*); no hybridization was observed outside end-plate regions. When sections were incubated with ε-subunit-specific sense probes, no hybridization could be detected (data not shown). These observations suggest that autoradiographic grain clusters reflect locally increased ε-subunit mRNA levels below the end-plate membranes. Similar results were obtained after hybridization with α-subunit-specific antisense (Fig. 1*d*, *e*) and sense probes and confirm the synaptic localization of α-subunit mRNA in rat muscle, as observed previously using northern blot analysis[5]. In some fibres, a small signal was observed above nuclei in the perijunctional region of the muscle fibres (Fig. 1*e*).

Previous northern blot analysis of AChR-specific mRNAs in neonatal rat muscle indicated that ε-subunit mRNA is barely detectable at birth but that levels increase rapidly during the first 2 weeks of postnatal development[4]. To determine whether this increase in ε-subunit mRNA is restricted to the end-plate region and therefore would be induced focally by the nerve, or whether the increase is more general, involving the entire fibre, we hybridized triceps muscle from rats of different postnatal ages with an ε-subunit mRNA-specific cRNA probe. Figure 2*a* shows the localization of AChE and autoradiographs of longitudinally sectioned muscle (*b*–*d*). At postnatal day 1, no hybridization signal could be detected either synaptically or extrasynaptically (Fig. 2*b*). In dark-field microscopy, some of the synaptic sites revealed a weak accumulation of grains (data not shown). However, on postnatal days 5, 9 (data not shown) and 12, an increasingly stronger signal was seen (Fig. 2*c*) that always coincided with the AChE-stained synaptic sites. Thus, the postnatal appearance of ε-subunit mRNA is restricted to the end-plate region from the earliest stages of synapse development and therefore must be induced by the nerve–muscle contact. As in adult muscle, hybridization signals in postnatal day-12 muscles were clearly associated with individual nuclei, as shown in Fig. 3. However, given the high density of nuclei from various cell types, unequivocal attribution to subneural nuclei was not always possible.

In contrast, total α-subunit mRNA remained at a plateau level during the first 12 postnatal days[4]. During this period, the α-subunit mRNA was detected throughout the fibre, in both the synaptic and extrasynaptic fibre segments (Fig. 2*f*, *g*). Although there were more grains at the synaptic sites, they were more widely distributed than those obtained upon hybridization with the ε-subunit mRNA specific probe. Moreover, the hybridization signal was also observed outside the myofibre bundles above unfused cells.

The level of total muscle ε-subunit mRNA increases almost normally in neonatal muscle denervated shortly after birth[4], indicating that only the brief, prenatal nerve–muscle contact is necessary to induce ε-subunit mRNA synthesis. We have investigated whether the ε-subunit mRNA still appears focally at the

Transgenic Research 12: 59–69, 2003.

Strain-dependent differences in the efficiency of transgenic mouse production

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Received 15 May 2002; revised 23 August 2002; accepted 26 August 2002

Key words: egg donor, mouse genetic backgrounds, mouse strains, pronuclear microinjection, transgenesis, transgenic mouse production

Abstract

Transgenic mouse production via pronuclear microinjection is a complex process consisting of a number of sequential steps. Many different factors contribute to the effectiveness of each step and thus influence the overall efficiency of transgenic mouse production. The response of egg donor females to superovulation, the fertilization rate, egg survival after injection, ability of manipulated embryos to implant and develop to term, and concentration and purity of the injected DNA all contribute to transgenic production efficiency. We evaluated and compared the efficiency of transgenic mouse production using four different egg donor mouse strains: B6D2/F1 hybrids, Swiss Webster (SW), outbred, and inbred FVB/N and C57BL/6. The data included experiments involving ~350 DNA transgene constructs performed by a high capacity core transgenic mouse facility. Significant influences of particular genetic backgrounds on the efficiency of different steps of the production process were found. Except for egg production, FVB/N mice consistently produced the highest efficiency of transgenic mouse production at each step of the process. B6D2/F2 hybrid eggs are also quite efficient, but lyze more frequently than FVB/N eggs after DNA microinjection. SW eggs on the other hand block at the 1-cell stage more often than eggs from the other strains. Finally, using C57BL/6 eggs the main limiting factor is that the fetuses derived from injected eggs do not develop to term as often as the other strains. Based on our studies, the procedure for transgenic mouse production can be modified for each egg donor strain in order to overcome any deficiencies, and thus to increase the overall efficiency of transgenic mouse production.

Introduction

Analysis of transgenic mice has become a key approach for studying the function of genes in the context of the whole organism, as well as for modeling human diseases. A centralized transgenic mouse facility provides an efficient and economical way to produce transgenic mice for a large number of researchers who are engaged in a wide variety of scientific disciplines, since expensive specialized equipment does not need to be purchased or the critical technical skills acquired. The present article addresses two subjects pertaining to the efficiency of transgenic mouse production: the effect of different genetic backgrounds of the egg donors on transgenic production, and what are the ideal measurements for evaluating transgenic mouse production.

The production of transgenic mice by injection of DNA into the pronucleus of a zygote became a firmly established technique during the 1980s, when

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optimized protocols for the procedure were published (Brinster et al., 1985; Hogan et al., 1986). The Induced Mutant Resources (IMR) database at Jackson Laboratories lists approximately 260 transgenic lines that are available for distribution, and hundreds of additional transgenic mouse lines are maintained in individual laboratories. In addition, many transgenic mice are not saved as lines after a study is completed. Consistent with this wide use of transgenic mice in biomedical research, a policy of the National Institutes of Health (US) is that the proportion of funds directed towards mouse models should rise to stimulate a substantial increase in the number of transgenic animals produced (Croy, 2000). In order to ensure that the transgenic mice are produced efficiently and in an economic manner, it is important to establish uniform criteria for evaluating the efficiency of transgenic production and to identify the key parameters that influence transgenic mouse production.

For many experiments involving the use of mice, the choice of genetic background is very important for the outcome (for example: Dandekar & Glass, 1987; Roudebush & Duralia, 1996; Scott & Whittingham, 1996; Sztein et al., 2000). To date, only a handful of papers have attempted to address factors that influence the efficiency of transgenic mouse generation and the choice of genetic backgrounds. Brinster and his colleges analyzed the efficiency of transgenic mouse production with regard to an optimized microinjection procedure, in terms of DNA concentration, size and form, the site of injection, and buffer composition (Brinster et al., 1985). The study also noted that generating transgenic mice with a hybrid line was eight times more efficient than with inbred C57BL/6 mice, and concluded that the overall efficiency of transgenic production can be influenced by the choice of mouse strains. Three other publications contain results of DNA microinjection using different strains, but less than one thousand eggs were manipulated in each case (Taketo et al., 1991; Canseco et al., 1994; Osman et al., 1997). With these small numbers there was considerable variation between studies. One more paper (Paris et al., 1995) reported efficiencies with FVB/N and two hybrid mouse lines that were ten fold lower than those shown by the other studies and attributed this to the transgenes used. Furthermore, in a technical guide for making mouse transgenics (Mann & McMahon, 1993) the authors provided results of manipulating eggs from their laboratory using only one donor strain (B6CBA/F1) and reported an unusually high efficiency of transgenic production, but did not point to the factor(s) that specifically promoted such a high result. Updated and more extensive analysis therefore is needed to determine the contribution of strain-dependent factors to the efficiency of transgenic mouse production.

In the present study we analyzed the results of injecting tens of thousands of eggs from four strains of mice. The data were collected over a four-year period to overcome the normal fluctuations seen in transgenic production due to subtle changes in laboratory conditions. Our studies enabled us to identify key characteristics of the four strains that significantly influence the efficiency of transgenic mouse production.

Materials and methods

DNA construct purification and quantification

Transgenic constructs were prepared in over 30 individual laboratories within NYU School of Medicine and were made according to our recommended protocol. DNA containing the expression cassette was separated from the vector and purified from an agarose gel slice by electroelution, followed by phenol/chloroform extraction and dialysis in TE (dialysis tubing from Invitrogene, previously Gibco BRL, #15961-022, Collodion bag from Sartorius Corp., #13200), or buffer exchange using Centriprep-30 concentrators (Millipore, #4306), followed by ethanol precipitation and washes in 70% ethanol. DNA was resuspended in microinjection buffer (MIB: 10 mM Tris pH 7.4, and 0.15 mM EDTA pH 8.0) at a concentration of at least 40 ng/μl. DNA was stored frozen at −20°C. The bacterial artificial chromosome (BAC) DNA purification method involved alkaline lysis, followed by double acetate precipitation of DNA and then purification of the BAC DNA on a CsCl gradient, followed by buffer exchange using Centriprep-30 concentrators. The samples were stored at 4°C in TE. Before injection, BAC DNA was diluted to ~0.3–2 ng/μl in MIB containing 30 μM spermine and 70 μM spermidine.

The transgenic facility staff analyzed the DNA by gel electrophoresis. For constructs smaller than 10 kb the DNA concentration, construct size and integrity (lack of smearing or additional bands) were evaluated by agarose gel electrophoresis. A series of sample dilutions were run on a 1% agarose gel with a DNA

ladder standard (High DNA Mass Ladder, Invitrogene, previously Gibco BRL, #10469-016) of a known amount of DNA for each band. For plasmid constructs larger than 10 kb, DNA concentration was measured using a fluorometer (Hoefer, DQ200) and an agarose gel was run to evaluate the integrity of the DNA. Only DNA constructs that were a single band on an agarose gel were used for injection. For BAC DNA, integrity was determined using pulsed field gel electrophoresis.

Generation of transgenic mice

DNA was injected into the male pronuclei of oocytes according to the procedure described in *Manipulating the Mouse Embryo* (Hogan et al., 1986). DNA was thawed and diluted to a concentration of 2–5 ng/μl on the day of injection. For one day of injection (defined as an experimental day) 15 females were superovulated and mated, embryos were dissected from plug positive females and all fertilized eggs were used for injections. The vast majority (90%) of experiments involved manipulation of at least 120 eggs (range: 90–250). The light cycle in the mouse rooms was 5 PM off and 3 AM on. Donor females were injected intraperitonealy with 5 IU pregnant mare's serum (PMSG, Calbiochem, #367222) at 3 PM and with 5 IU human chorionic gonadotropin (hCG, Pregnyl, Organon Inc., #0052-0315-10) 46 h later (1 PM) and immediately mated with appropriate stud males. The males were monitored for their breeding performance by keeping a record of plugging. Males that did not mate on several occasions (4–6) were removed from the facility. For C57BL/6 males, an additional test was performed which involved placing a female in the cage and checking whether the male failed to fertilize the female after two weeks (lack of implantation sites in the uterus). We observed that the frequency of mating is strongly influenced by two factors: how often males are used and the age of donor females. Once we optimized these two factors there was no difference in the plug rate between strains.

Microinjections were performed by four experienced technicians with an Eppendorf Transjector 5246. After injection of DNA into the pronucleus, embryos were cultured overnight (17–26 h) in M16 medium (Specialty Media, MR-016-D) at 37°C and 6–7% CO_2, unless otherwise noted in the results. Hormones, media and culturing conditions were routinely monitored for optimal quality by recording the number of embryos obtained on a daily basis and by periodic controls involving culture of non-manipulated zygotes to the blastocyst stage, followed in some cases by embryo transfer. The day after pronuclear injection the embryos that had divided to the 2-cell stage were implanted into Swiss Webster (SW) foster mothers. Approximately 15 embryos were transferred into each oviduct of each recipient. In a few cases ~35 embryos were transferred into one female. In the results presented, we included experiments in which one of the recipients from an experiment died. The number of embryos that lyzed after the injection procedure was recorded. Similarly, the number of embryos that underwent a 1-cell block was recorded. Progeny were genotyped by PCR and/or Southern blot analysis in individual investigator's laboratories. In ~15% of the experiments founder embryos were examined for lacZ expression by X-Gal staining and not genotyped, thus the actual number of transgenics was likely higher.

Mice

Outbred SW, hybrid B6D2/F1, and inbred FVB/N, C57BL/6 or 129S6/SvEv mice were purchased from Taconic Farms. F2 hybrid zygotes for manipulation were obtained by interbreeding B6D2/F1 mice.

Databases

Custom designed FileMaker Pro databases, kindly provided by Tom Clarke (NYU Kaplan Comprehensive Cancer Center), were used for weekly scheduling, record keeping, and summarizing results. The weekly scheduling database allowed technicians to sign up for superovulated females from each of the four strains, and naturally mated or pseudopregnant females. Signing up was organized by plug detection date. The database generated a weekly schedule with dates for hormone injections, animal mating and plug checking. To keep track of the results of microinjection experiments a separate microinjection datasheet was used for each construct. The datasheet included general data such as: laboratory submitting the request, the DNA construct name and size, individual laboratory and core facility evaluation of DNA sample concentration, and dilution used for microinjection. Each row of the table represented one experimental day. For each experimental day the number of plug positive females was recorded, as well as the number of fertilized eggs recovered, number of eggs injected, number of eggs that lyzed, or underwent 1-cell blocks, date of embryo transfer, number of eggs transferred, number of pseudopregnant females used for the transfer, number of females which got pregnant, number of pups

Table 1. Quantity and quality of embryos obtained from superovulated females by donor strains. Significance evaluated by the standard *Z*-test

Donor strain	Average no. of fertilized eggs/ experiment[a]	STD error of means	Egg survival = % total transferred[c] / total eggs injected	Observations
FVB/N	149.88[b]	3.27	80.26	Inconsistent response to hormones, large pronuclei, eggs advance quickly to 2-cell stage, frequent mosaic founders
B6D2/F1	167.88[b]	3.11	72.55	Consistent number of fertilized eggs with accessible pronuclei
SW	148.01[b]	2.38	61.38	Large fraction of poor quality embryos
C57BL/6	155.92	7.72	71.32	Asynchronous and slow development of eggs with small poorly visible pronuclei

[a] Fifteen females were superovulated and mated (rate of mating can be considered as part of breeding performance of the strain).
[b] Statistically significant difference between B6D2/F1 and SW or FVB/N (at 1% level).
[c] Subjective evaluation of suitability of embryos for transfer into recipients was applied in which all 2-cell stage embryos were used and some elongated 1-cell stage embryos were also transferred.

born, and number of transgenics reported by the individual laboratory. Any unusual observations were also recorded.

Statistical evaluation

Significance of the differences between results was evaluated by the standard *Z*-test based on the normal distribution.

Results

The data used for the studies presented is from all experiments conducted in a period of four years. Over this period, 346 DNA transgene constructs were injected into zygotes on 797 experimental days. Eggs with four different genetic backgrounds were used (B6D2/F2 hybrid, FVB/N or C57BL/6 inbred, and SW outbred) and 2268 transgenic mice were produced. The results of seemingly failed experiments (e.g., a large proportion of the injected eggs did not survive manipulation, a very low number of babies were born, or some of the recipients did not give birth) were included in the data to allow us to investigate the reasons for such unfavorable outcomes. The overall transgenic mouse production efficiency is expressed as a percentage of the number of transgenics obtained from the number of eggs injected.

Strain-dependent effects on donor egg production

A major factor influencing transgenic mouse production is the quality of donor eggs and the efficiency of producing them. Table 1 summarizes the results of egg production for four donor strains. Fifteen females were superovulated for each experimental day and the average daily number of fertilized eggs that were produced for each genetic background was calculated. Statistically significant differences were identified between B6D2/F1 and FVB/N or SW donor mice, with B6D2/F1 hybrid intercrosses producing a higher number of fertilized eggs per day than FVB/N or SW (~168 v.s. ~150 and ~148, respectively). C57BL/6 mice produced an intermediate number of donor eggs (~156).

Tolerance of eggs to DNA microinjection

Egg survival was evaluated only for experiments in which embryo transfers were performed on the day after injection. An egg survival percentage was calculated for each strain as the number of eggs that survived overnight culture and were suitable for transfer into recipients (in most cases the eggs had divided to the 2-cell stage), from the total number of fertilized eggs that were injected. Table 1 presents the results of injection of tens of thousands of eggs. FVB/N eggs seemed to have the highest egg survival (~80%), whereas SW eggs had the lowest survival rate (~60%) between the four strains.

To identify factors that lower egg survival, both loss of injected eggs due to lysis soon after injection and due to a block in cell division (1-cell block) were evaluated. We detected a significantly higher rate of lysis for B6D2/F2 eggs (~22%) compared to FVB/N and C57BL/6 eggs (~16 and ~18%, respectively). A

Table 2. Results of DNA microinjections by donor strain. Significance evaluated by the standard Z-test

Donor strain	No. of eggs injected	% Eggs lyzed/ injected	% 1-Cell blocks/ injected	% Born/ injected	% Born/ transferred[e]
FVB/N	28,608	15.73[a]	4.41	16.34[c]	20.28
B6D2/F1	30,369	22.10[a]	5.12	13.36[c]	18.56
SW	54,027	20.85	17.87[b]	11.67	19.43[d]
C57BL/6	7139	17.84[a]	10.86	9.43[c]	13.87[d]

[a] Significantly higher for B6D2 than for FVB/N or C57BL/6 (at 1% level).
[b] Significantly higher for SW than for other three strains (at 1% level).
[c] Significantly lower for C57BL/6 than for FVB/N or B6D2/F1 (at 1% level).
[d] Significantly lower for C57BL/6 than for SW (at 1% level).
[e] Subjective evaluation of suitability of eggs for transfer into recipients was applied. All 2-cell stage embryos and some elongated 1-cell stage embryos were transferred.

high percentage of SW egg lysis (~21%) was also observed. What was striking, however, was that the number of SW eggs that blocked at the 1-cell stage following DNA injection was much higher (at 1% level) compared to FVB/N, B6D2/F2 or C57BL/6 eggs (~18% v.s. ~4%, ~5% and ~11%, respectively; see Table 2).

All eggs that were at the 2-cell stage after overnight culture were transferred into SW foster mothers on the next morning. The percentage of pups born from the total number of injected eggs was significantly lower (at 1% level) for C57BL/6 (~9%) than for FVB/N (~16%) and B6D2 hybrids (~13%). There also was a significant difference in the percentage of pups born from the number of eggs transferred between C57BL/6 and SW. By using the number of embryos transferred rather than injected, the influence of egg loss due to lysis or 1-cell blocks is not included in the measurement. The frequency of pregnancy was not found to vary greatly between the four strains of eggs used (80–91%), based on visual inspection of recipients at mid-gestation, thus this cannot be the primary reason for the low number of mice born for C57BL/6 2-cell stage embryos. The pregnancy rate for injected C57BL/6 embryos (~85%) was only slightly lower than for FVB/N embryos (~91%). Furthermore, some of the female recipients of C57BL/6 manipulated eggs thought to be pregnant, but which failed to deliver, showed resorption sites when autopsies were performed soon after the due dates. Consistent with a loss of embryos during late development in utero, in a control experiment in which C57BL/6 embryos were transferred into recipients without DNA injection or overnight culture, only ~28% developed to term, although the majority of the mothers gave birth (~86%).

Evaluation of transgene DNA quality

We found that the quality of DNA prepared for microinjection can greatly influence the overall production efficiency. Over the four-year period of operation of the core facility some laboratories consistently provided DNA that was more 'sticky' or 'difficult' to inject compared to other laboratories. The ease with which a transgene could be injected was recorded in the microinjection datasheet. We observed six cases in which the first sample of a DNA construct caused a high number of 1-cell blocks after overnight culture and produced no transgenics, whereas an independently purified second sample of the same construct produced few 1-cell blocks and transgenic mice. In these cases the difficulties could be attributed to the quality of the DNA sample, rather than to a specific construct. Experiments with all DNA preparations are included in the data presented.

As a possible means to evaluate the influence of DNA purity on transgenic production we compared the results obtained from each of two laboratories that made a large number of transgenic constructs with the cumulative results of nearly 20 laboratories that made transgenic constructs less frequently (Table 3). To remove the variable of genetic background of the egg donor, results from one strain (SW) were compared. Group A contained results of 53 constructs from 18 different laboratories submitting constructs infrequently (1–8 constructs/laboratory), group B contained results from one laboratory for 37 injected constructs and group C contained results from yet another laboratory for 29 constructs injected. The overall transgenic production efficiencies (% transgenics of eggs injected) for the two more experienced groups were higher (at 1% level) than for the inexperienced

Table 3. Comparison of transgenic production efficiencies expressed as percentage of transgenics obtained from eggs injected for laboratories with different experience in making transgene DNA. Significance was evaluated by the standard Z-test

Strain	Group (no. of laboratories)	No. of constructs	% Eggs lyzed/ injected	% 1-Cell blocks/ injected	% Born/ injected	% Transgenics/ eggs injected
SW	A (18)	53	21.1[a]	20.5[b]	10.7[c]	1.5[d]
	B (1)	37	18.4[a]	15.3[b]	14.2[c]	2.1[d]
	C (1)	29	19.8	19.0	12.8	2.3[d]
FVB/N	D (19)	57	15.7	4.4	15.6	2.7[e]
	E (1)	19	15.2	4.6	19.2	4.5[e]

[a] Statistically significant difference between two groups. Results for group A that made transgenic constructs less frequently were higher than for experienced group B (at 5% level).
[b] Statistically significant difference between two groups. Results for group A were higher than for group B (at 1% level).
[c] Statistically significant difference between two groups. Results for group A were lower than for group B (at 1% level).
[d] Statistically significant difference in results for laboratories that made a large number of transgenic constructs (groups B and C) than for group A (at 1% level).
[e] Statistically significant difference between two groups. Results for experienced group E were higher than for group D that made transgenic constructs less frequently (at 5% level).

group (A – 1.5%; B – 2.1%; C – 2.3%). Similarly, we analyzed the overall transgenic production efficiency for laboratories using FVB/N egg donors. Group D contained results of 57 constructs from 19 different laboratories that produced transgenic constructs infrequently (1–8 constructs) and group E contained results from one laboratory for 19 constructs. The overall results of groups D and E were significantly different at 5% level (D – 2.7%; E – 4.5%). In an attempt to determine at what step of the procedure poor DNA impedes the outcome we statistically analyzed each step of transgenic production for both strains and found that for the SW background that the less experienced laboratories were less efficient in all steps than one of laboratories producing transgenic mice more frequently. This suggests that poor quality DNA may affect each step of production with the cumulative lowering of the overall transgenic production efficiency.

Influence of time of embryo transfer on transgenic production

Most of the published papers relating to procedures for transgenic mouse production (Hogan et al., 1986; Mann & McMahon, 1993; Pinkert, 1994; Hammes & Schedl, 2000) describe transferring the embryos to recipient mothers either soon after the DNA injection is performed, or on the next morning. Mann and McMahon (1993) reported similar efficiencies of transgenic production for both times of embryo transfer for hybrid eggs. The majority of DNA microinjection experiments in our data set involved transferring the embryos on the morning after the DNA injection. On some occasions, however, eggs were transferred on the same day. We compared the results of 28 experiments in which eggs were injected with the same construct and transferred on the same day or transferred on the next day (Table 4). For SW egg donors the percentage of pups born from the injected eggs was significantly higher (at 5% level) when the eggs were transferred on the same day as the DNA injection compared to on the next day (14.4% v.s. 9.7%). Surprisingly, the percentage of transgenic pups produced from the number of eggs injected was also significantly (10% level) higher when eggs were transferred on the day of microinjection rather than on the next day (2% v.s. 1.1%, respectively). This suggests that the uterus provides a more optimal environment than *in vitro* culture, specifically for transgenic eggs. For FVB/N and hybrids no statistically significant differences were observed when injected eggs were transferred to recipients on the same or next day.

Measurements of transgenic production efficiency

In Table 5 we show three different ways of evaluating the transgenic production efficiency in the

Table 4. Comparison of the efficiency of transgenic production using two times of embryo transfer. Significance is evaluated by the standard Z-test

Donor strain	Same day embryo transfer			Next day embryo transfer		
	No. of eggs injected	% Born/ injected	% TGs/ injected	No. of eggs injected	% Born/ injected	% TGs/ injected
FVB/N	1244	18.9	4.7	849	17.3	3.6
B6D2/F1	841	15.0	2.0	963	15.3	1.7
SW	1970	14.4[a]	2.0[b]	1877	9.7[a]	1.1[b]

[a] Significant difference of results between two times of transfer (at 5% level).
[b] Significant difference of results between two times of transfer (at 10% level).
Note lack of significant differences between two times for FVB/N and hybrid strain.
TGs: transgenics.

Table 5. Overall efficiency measurements of DNA microinjections by donor strains. Significance evaluated by the standard Z-test

Donor strain	No. of TGs/ experimental day	% TGs/ injected eggs	Maximum % TGs/ injected eggs obtained	% TGs/ born[b]
FVB/N	4.1	3.0[a]	17.63	17.4
B6D2/F1	3.3	2.1[a]	5.36	16.5
SW	2.1	1.7[a]	8.11	15.1
C57BL/6	1.8	1.2[a]	3.37	14.9

[a] All values significantly different (at 1% level).
[b] TGs: transgenics. Note lack of statistically significant differences.

four different genetic backgrounds: (i) the number of transgenic mice produced per experimental day; (ii) the percentage of transgenics produced from the number of eggs injected; and (iii) the percentage of transgenics produced from the number of animals born. For our facility, which has a user fee based on a defined injection day; it can benefit the customer to use a generic term 'the number of transgenics obtained per experimental day'. This number allows investigators to anticipate the cost of transgenic production depending on the number of transgenic animals required. The overall transgenic production efficiency measured as the percentage of transgenic animals produced from the number of eggs injected was found to be significantly different for all strains (at 1% level). The highest overall transgenic production efficiency (3.0%) was obtained with FVB/N eggs. Furthermore, the transgenic production rate was higher for B6D2/F2 hybrid eggs (2.1%) than for SW (1.7%) and C57BL/6 (1.2%) eggs. Of interest, there were no statistically significant differences in the percentages of transgenic mice produced from the number of mice born between the four strains. Thus, the main limitations to transgenic production come not from the injection procedure (once the procedure is optimized) or the frequency of DNA integration between different strains, but from embryo viability and resistance to the injection procedure and culture conditions. The efficiency of egg production is also a consideration when comparing FVB/N, SW and B6D2/F1 mice and this is reflected in the number of transgenics made per experimental day.

Discussion

There are many factors that influence the efficiency of transgenic mouse production and previous studies suggested that the genetic background of the egg donor could influence the overall efficiency of transgenic production (Brinster et al., 1985; Taketo et al., 1991; Mann & McMahon, 1993; Paris et al., 1995; Osman et al., 1997). In the present studies we have extended these findings considerably by analyzing a much larger data set and directly comparing four different mouse strains. Our studies allowed us to identify particular steps in the production of transgenic mice that

are significantly different between strains; response of egg donor females to superovulation, viability of eggs following injection, tolerance of injected eggs to overnight culture, and the ability of embryos to implant and develop to term.

Certain measurements of transgenic production efficiency can identify strain-dependent factors

To evaluate the efficiency of transgenic production in detail, several measurements should be considered (Tables 1, 2 and 5) to uncover the cause(s) of a lower than expected transgenic production efficiency. A commonly used measurement of the efficiency of transgenic production is the percentage of transgenic mice obtained from the number of animals born (or weaned). Such a measurement, however, does not reflect the efficiency of all the steps in transgenic production. Since every step influences the cost and effort required in producing transgenic mice, it is important to evaluate all contributing factors.

Each measurement of the efficiency of transgenic production takes into account the influence of a different subset of all the strain-dependent factors. Evaluation of transgenic production efficiency as the percentage of eggs injected measures a wider range of the processes, but still does not reflect the efficiency of embryo production (female response to superovulation and male fertility). A strong argument for expressing the efficiency of transgenic production with respect to a defined experimental day (15 females superovulated, all fertilized diploid eggs used), or to the number of eggs injected, comes from our finding that there is no significant difference in the percentage of transgenics produced per animal born between the four genetic backgrounds tested. This suggests that the frequency of DNA integration is the similar in different strains. However, when the efficiency of transgenic mouse production is expressed as a percentage of eggs injected, a significant difference was seen between all four genetic backgrounds. Thus, expressing transgenic production efficiency as a percentage of the eggs injected for the same strain is a more informative approach for comparing the results obtained by different laboratories or facilities. Calculating the percentage of transgenic mice obtained from the number of mice born, however, can be an important parameter, if it is expected that a transgene could cause lethality.

Using as an efficiency measure the number of transgenics obtained per standardized injection day or per total number of superovulated donor females used (see Materials and methods) allows the influence of all factors to be taken into account and presents a very good and simple tool for quick evaluation. If a core Transgenic Facility has a charge based on each injection day, it is also a good tool to evaluate expenses. However, if the production efficiency appears suboptimal, then the other measurements are useful for pinpointing where the problem is. Finally, one additional aspect that should be considered is the sensitivity of the genotyping assay. Since a good proportion of transgenics can be mosaic, it may be desirable to be able to detect weak mosaic transgenics and this may require PCR rather than Southern blot analysis. Depending on the genotyping procedure the true transgenic production efficiency rate of the facility might be higher than recorded. We have found this to be of particular importance for BAC transgenics in which the transgene copy number is low (A.L.J., unpublished observations).

One factor that is not strain dependent is the amount of the DNA injected into an egg. When more DNA is injected, it is often found that fewer pups are born, but a higher percentage of transgenics is obtained per animal born. We have observed as many as 70% transgenics using this measure. However, the total number of transgenic mice obtained from one experimental day can be lower when a higher concentration of DNA is injected.

Quality of transgene DNA has a major influence on transgenic production

We found that DNA transgenes purified according to the same protocol in some cases produced different efficiencies of transgenic production. We think this likely reflects a difference in the purity of the DNA, since the lower efficiencies were associated with a high rate of egg lysis and 1-cell blocks. Toxicity of DNA can be a major cause of variability in transgenic production efficiencies, when eggs from one donor strain are used. Such toxicity could result from such things as traces of phenol or ethanol, the presence of bacterial endotoxins, or from particles.

Our preliminary results (13 experiments with ~2000 eggs) using supercoiled BAC DNA injected into FVB/N eggs showed that injection of BAC DNA can produce nearly as many transgenic mice per egg injected (~2%) as plasmid-based constructs (~3%) with the same mouse strain, indicating that the size and form of a DNA construct can have little influence on the overall efficiency of transgenic production.

The key therefore is the purity of the DNA sample. For BAC constructs it is important to add polyamines to produce supercoiled DNA (Montoliu et al., 1995). Even with this BAC DNA purification method, we found that the number of 1-cell blocks was three times higher when BAC DNA was injected into zygotes (~12%) compared to plasmid-based constructs (~4%) for the same strain. Based on this, it is likely best to choose a strain with a low percentage of 1-cell blocks for making BAC transgenics.

Time of embryo transfer influences transgenic production efficiency only with some strains

There are a number of advantages of performing the embryo transfer procedure on the day after DNA injection. For instance, it allows time for rest after an afternoon of DNA injections and before the delicate procedure of transferring embryos into the oviducts of foster mothers is performed. Such timing also allows for a more precise estimate of the number of recipients that is needed for an experiment, based on the number of eggs that are actually injected. Furthermore, the number of embryos that block at the 1-cell stage can be determined, which can pinpoint problems with a particular DNA sample. However, for some strains it is better to do the embryo transfer on the same day as the DNA injection. Significantly, we found that SW eggs undergo a higher percentage of 1-cell blocks than other strains (Table 2) when they are cultured overnight after DNA injection, and that by transferring the eggs on the same day as the DNA injection this limitation was overcome (Table 4).

FVB/N mice produce the highest efficiency of transgenic production

Making transgenic founder animals in a defined inbred genetic background can be critical for experiments in which the genetic background is expected to influence the phenotype. The FVB/N strain is the most commonly used inbred strain for transgenic production because of its superior reproductive performance and prominent pronuclei, which facilitates microinjection of DNA (Taketo et al., 1991). In addition, in our hands FVB/N eggs develop in a synchronized manner. In our comparison of four strains, FVB/N embryos were found to have the highest efficiency of transgenic production when calculated either as a percentage of the eggs injected, eggs transferred, or number of transgenic mice produced per experimental day. We observed the previously reported (Taketo et al., 1991) high tolerance of FVB/N embryos for manipulation as reflected by the low number of eggs that lyzed or blocked at the 1-cell stage after injection. In general, we also noted a small range of variation with this strain, although the response of FVB/N females to superovulation was not always consistent (a range of 90–250 eggs were produced per experimental day). Of importance, egg production appeared to be particularly sensitive to the age of the donor, with 5-week-old females responding less well to superovulation than 4-week-old females.

There are some limitations to using FVB/N mice for phenotype studies, because they harbor two known mutations. One mutation causes retinal degeneration due to insertion of a provirus into the *Pde*b gene, which encodes the ß subunit of cGMP phosphodiesterase (Bowes et al., 1993; Gimenez & Montoliu, 2001). The mutation (*Pde*b^{rd1}, previously *rd*) results in postnatal rod photoreceptor degeneration that causes severe visual impairment (FVB/N are functionally blind). One substrain maintained by the APA at NCI-FCRDC, FVB/NCr, also is afflicted by a neuroendocrine syndrome ('Space cadet' syndrome). This syndrome seems to have arisen within the past six years (Hsiao et al., 1995; Ward et al., 2000) and leads to development of neuronal necrosis in the brain and liver, with associated behavioral changes.

B6D2/F1 hybrid intercrosses produce a large number of eggs, but they are prone to lysis

Hybrid mouse lines have been used extensively for generating transgenic mice because of their superior breeding performance and efficiency of transgenic production. Typically, one of the parental inbred strains is C57BL/6 and F1 parents are used because they show hybrid vigor. The main advantage we found of using B6D2 intercrosses to produce eggs was that the superovulated females produced more eggs than the other three strains analyzed. A disadvantage of the F2 hybrid eggs is that they contain smaller pronuclei than FVB/Ns. Like FVB/N eggs, the B6D2/F2 eggs develop in a synchronized manner. However, B6D2/F2 egg survival after injection is lower than that for FVB/N due to a higher percentage of egg lysis after DNA injection.

Injected SW eggs block at the 1-cell stage at a high rate

For experiments in which the genetic background is not important (for example analysis of gene regulatory

elements), outbred strains (for example SW, CD-1, ICR) can be used to produce transgenics. However, we found that SW mice produce less transgenic animals per experimental day than FVB/N or hybrids (Table 5). The cost per transgenic mouse is therefore not lower using outbred SW mice than inbred FVB/N. The key factor that lowers the overall efficiency of transgenic production with SW mice is a high rate of 1-cell blocks. We found that SW zygotes injected with DNA blocked at the 1-cell stage four times more often than the other strains. Since we found that non-injected SW eggs cultured overnight did not block at the 1-cell stage at such a high rate (< 2% v.s. ~18% for injected), SW eggs are not the best choice for microinjection of DNA, such as BAC transgenes, which cause excessive egg damage.

C57BL/6 eggs produce fewer pups and transgenics than other strains

C57BL/6 mice have been extensively characterized genetically and are popular in many research studies. We were therefore interested in determining the optimal conditions for producing transgenics with C57BL/6 eggs. As discussed above, the percentage of transgenics produced from the number of mice born was not significantly different between C57BL/6 and the other three strains. Despite this, however, nearly every aspect of transgenic production was found to be less efficient, or more difficult, with C57BL/6 mice than with FVB/N or hybrid mice. C57BL/6 mice are poor breeders and the mothers often abandon their pups. Since C57BL/6 eggs contain granular pigmentation, their pronuclei are less visible and are more difficult to inject. The skill of the person doing the DNA injection can therefore play a critical role in the efficiency of transgenic production for C57BL/6 mice than for others. We also found that C57BL/6 preimplantation embryos often develop slower than other embryos, requiring that the DNA injections be performed later in the afternoon or the superovulation schedule be shifted ahead of the other strains. In addition, we found that C57BL/6 embryos develop asynchronously. Despite these limitations, C57BL/6 eggs tolerate DNA injection and embryo transfer well. Egg survival (Table 1) was similar to that for hybrids and better than for SW. However, the ability of C57BL/6 eggs to continue to develop to term after implantation was impaired compared to the other three strains, resulting in a low number of pups being born. Significantly, our finding that C57BL/6 transgenic mice can be produced at a frequency of ~2 transgenics per experimental day shows that it is worthwhile making transgenics in C57BL/6 eggs, rather than using hybrid mice and backcrossing to obtain an inbred background.

We performed a limited number of injections into yet another inbred strain, 129S6/SvEv (776 eggs injected; data not shown). We obtained a low (below 1%) percentage of transgenics from injected eggs (see Table 5 for other strains). The low efficiency seemed to result from poor egg survival after DNA injection (a high number of 1-cell blocks), and a low number of fetuses surviving to birth. Nevertheless, it is possible to produce transgenics with 129S6/SvEv mice.

Conclusion

Our analysis of the factors that influence transgenic production in four different strains of mice have uncovered the key steps that should be considered when making transgenics in a new strain, and has identified strain-dependent factors for each of the four strains analyzed. We have shown that the number of transgenics produced per defined experimental day, or per number of superovulated donors, is the best measurement of the transgenic production efficiency to use when comparing efficiencies in different facilities, because it takes into account all aspects of the production. Transgenic production efficiency expressed as the percentage of the transgenics obtained from the number of eggs injected is a good measure for pinpointing problems, although it does not take into consideration embryo production. By comparing the performance of four mouse strains at all steps of transgenic production, we have been able to modify the procedure for SW and C57BL/6 mice to overcome strain-dependent characteristics that decrease the overall efficiency of transgenic mouse production.

Acknowledgements

The National Cancer Institute's Cancer Center Support Grant, P30 CA16087, and the Skirball Institute of Biomolecular Medicine provided support for this project. The authors thank D. Osborn for excellent technical assistance, and the many laboratories of NYUSM that provided transgenes and founder results. Alexandra Joyner is an investigator of the HHMI.

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Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9302–9306, September 1994
Biochemistry

Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter

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Communicated by Philip Leder, May 5, 1994

ABSTRACT **Promoters whose temporal activity can be directly manipulated in transgenic animals provide a tool for the study of gene functions *in vivo*. We have evaluated a tetracycline-responsive binary system for its ability to temporally control gene expression in transgenic mice. In this system, a tetracycline-controlled trans-activator protein (tTA), composed of the repressor of the tetracycline-resistance operon (*tet* from *Escherichia coli* transposon Tn*10*) and the activating domain of viral protein VP16 of herpes simplex virus, induces transcription from a minimal promoter ($P_{hCMV*-1}$; see below) fused to seven *tet* operator sequences in the absence of tetracycline but not in its presence. Transgenic mice were generated that carried either a luciferase or a β-galactosidase reporter gene under the control of $P_{hCMV*-1}$ or a transgene containing the tTA coding sequence under the control of the human cytomegalovirus immediate early gene 1 (hCMV *IE1*) promoter/enhancer. Whereas little luciferase or β-galactosidase activity was observed in tissues of mice carrying only the reporter genes, the presence of tTA in double-transgenic mice induced expression of the reporter genes up to several thousand-fold. This induction was abrogated to basal levels upon administration of tetracycline. These findings can be used, for example, to design dominant gain-of-function experiments in which temporal control of transgene expression is required.**

Functions of mammalian gene products in development and oncogenesis have been defined by their actions in dominant gain-of-function experiments in transgenic animals (1). The transgenes in these experiments are controlled by either tissue-specific or ubiquitously expressed promoters. Their temporal and spatial expression patterns are dependent upon the characteristics of the promoters employed. However, numerous questions require control over timing and tissue-specific expression of a transgene.

Although several inducible systems have been established in transgenic mice, all have limitations. In the classic binary systems, the target gene is silent and can be activated upon crossing in a transgene that encodes either a trans-activator or a recombinase (2–4). In such systems, the temporal activity of the target gene is dependent on the expression pattern of the effector molecule (trans-activator or recombinase), and it cannot be directly regulated by changing experimental conditions. In systems based on environmental signals such as steroid hormones or heavy metal ions, gene expression can be modulated (for review, see ref. 5). However, generalized physiologic or toxic effects from the inducing chemicals and high basal-transcriptional activity from the promoters limit their utility. Finally, tissue-specific and hormone-inducible promoters, such as the long terminal repeat of mouse mammary tumor virus or the whey acidic protein gene promoter, direct gene expression to only a few selected tissues, and the timing of gene expression is primarily controlled by endogenous hormone levels (6, 7).

Yet another approach to control gene expression has been to adopt well-characterized regulatory systems from *Escherichia coli* for use in mammalian cells (8). Transgenic systems based on the *lac* operon have proven inadequate because of inefficient induction levels and kinetics (5). However, the development of a regulatory circuit based on the tetracycline-resistance operon *tet* from *E. coli* transposon Tn*10* opened a new approach to controlling transgene expression (9, 10). In this system, a fusion tetracycline-controlled trans-activator protein (tTA) composed of the *tet* repressor and the activating domain of viral protein VP16 of herpes simplex virus strongly activates transcription from $P_{hCMV*-1}$, a minimal promoter from human cytomegalovirus (hCMV) fused to *tet* operator sequences. The tTA binds to the *tet* operator sequences in the absence of tetracycline but not in its presence. This results in repression of transcription upon introduction of tetracycline. In the animal, tetracycline derivatives are readily absorbed and broadly distributed to different tissues with minimal toxicity at the concentration needed to regulate the activity of the synthetic promoter (11).

In this study we have evaluated the tetracycline-responsive regulatory system as a means to temporally regulate transgene expression in animals. The luciferase reporter gene was used as a sensitive measure of expression levels in whole tissues, and the β-galactosidase gene was used to monitor expression at the single cell level *in situ*.

MATERIALS AND METHODS

Generation of the Transgenes and Transgenic Mice. The tTA-encoding sequence contained in plasmid pUHG15-1 (P.G. and H.B., unpublished data) is under the control of the hCMV *IE1* promoter/enhancer ("hCMV-tTA gene") and is flanked at the 3′ end by the rabbit β-globin intron and a poly(A) signal. Plasmid pUHC13-3 containing the luciferase gene has been described (9). The β-galactosidase reporter gene containing a nuclear localization signal was constructed as follows: an *Xba* I–*Bgl* II fragment, containing the nuclear β-galactosidase structural gene, an intron, and a poly(A) signal, was excised from plasmid pNlacF (12) and cloned into a plasmid containing $P_{hCMV*-1}$. The β-galactosidase-encoding transcription unit was separated from the vector with *Xho* I and *Bgl* II and isolated as a 4.3-kb fragment from an agarose gel by using electroelution. The hCMV-tTA gene was isolated

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Abbreviations: rlu, relative light unit(s); tTA, tetracycline-controlled trans-activator; hCMV, human cytomegalovirus.

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as a 2.7-kb *Xho* I–*Pfl*mI fragment, and the luciferase reporter gene was isolated as a 3.1-kb *Xho* I–*Eae* I fragment.

The DNA fragments were injected into fertilized eggs at a concentration of ≈5 ng per μl. Transgenic mice were generated according to standard procedures, and founder mice were analyzed by using the PCR and Southern hybridization. The tTA-encoding transgene was identified by using primers corresponding to the hCMV promoter from −50 to −33 (5′-GGC GTG TAC GGT GGG AGG-3′) and sequences encoding the *tet* repressor (5′-GCA AAA GTG AGT ATG GGT CC-3′). The resulting PCR product was 280 bp in size. The reporter genes were identified with primers corresponding to the hCMV promoter (see above) and the luciferase gene (5′-GCA ATT GTT CCA GGA ACC AGG GCG-3′) or the nuclear localization signal of the β-galactosidase gene (5′-CGG GAT CCC CCA TGC TCC CC-3′). The PCR product for the luciferase gene was 320 bp long and that for the β-galactosidase transgene was 269 bp long. Three types of transgenic mice were generated: mice that carried the hCMV-tTA gene, the luciferase reporter gene, or the β-galactosidase reporter gene containing sequences encoding a nuclear translocation signal.

Administration of Tetracycline. Slow-release tetracycline pellets (Innovative Research of America) were implanted subcutaneously in the shoulder region using a trochar according to the manufacturer's directions. These pellets released 0.7 mg of tetracycline hydrochloride per day. All pellets were kept in place for 7 days before levels of transgene expression were measured. Transgene expression following tetracycline withdrawal was measured 7 days after pellet removal. Tetracycline pellets were given to five females 1–4 days prior to mating. All females became pregnant, and a total of 50 normal pups were delivered. No toxicity from the tetracycline was seen.

Analysis of Luciferase and β-Galactosidase Activities. To analyze luciferase activity, mice were killed by cervical dislocation, and tissue samples were homogenized by using a Polytron in lysis buffer containing 25 mM glycylglycine, 15 mM Mg_2SO_4, 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100. The homogenate was centrifuged for 5 min at 12,000 rpm (14,000 × *g*), and 100 μl of the supernatant was added to 350 μl of assay buffer (25 mM glycylglycine, 15 mM Mg_2SO_4, 5 mM ATP). Luciferase activity was measured using a Berthold Lumat luminometer (Berthold, Germany) after the injection of 100 μl of a 0.05 mM luciferin solution. The protein concentration of the homogenate was determined by using the Bradford assay (Pierce Coomassie protein assay). Luciferase activities were calculated as relative light units (rlu) per mg of total cellular protein.

β-Galactosidase activity was assayed in whole-tissue samples or in frozen sections. To analyze activity in whole-tissue samples, 5-mm cubes of selected tissues were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in phosphate-buffered saline (PBS) for 1 hr and then rinsed twice in PBS. Staining for β-galactosidase activity was done at 30°C in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl β-D-galactopyranoside, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6{\cdot}6H_2O$. After staining, the specimens were embedded in paraffin, 10-μm sections were cut and counterstained with eosin or nuclear fast red, and an examination for blue-colored nuclei was conducted. To analyze β-galactosidase activity during embryogenesis, embryos were fixed for 12 hr at 4°C in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 0.2% Nonidet P-40, and 2.5 mM deoxycholic acid. Embryos were then bisectioned and stained as described above.

RESULTS

Generation of Transgenic Mice. Three types of transgenic mouse lines were generated: reporter mice containing either the luciferase or the β-galactosidase transcription unit and mice carrying the hCMV-tTA gene. The hCMV *IE1* promoter/enhancer was chosen because it is expressed in a broad spectrum of tissues in transgenic mice (13, 14). The reporter genes, encoding either luciferase or the bacterial β-galactosidase, were under the control of $P_{hCMV*-1}$. Whereas the luciferase reporter gene permitted rapid and sensitive analysis of overall transgene expression in selected organs, the β-galactosidase reporter gene enabled us to specifically identify the expressing cell types. A nuclear localization signal in the transgenic β-galactosidase allowed us to easily distinguish it from endogenous cytoplasmic β-galactosidase activity (12). From the six founder animals carrying the hCMV-tTA gene, five (TA1–TA5) were used to establish lines. From the 13 founder mice carrying the luciferase gene, 6 (LU1–LU6) were used to establish lines, and lines were established from the 4 founder animals (G1–G4) carrying the β-galactosidase gene. Double-transgenic mice carrying the hCMV-tTA gene and one of the two reporter genes were generated through cross breeding.

The Luciferase and β-Galactosidase Genes Were Activated by tTA. Basal expression levels of the luciferase gene and the magnitude of induction by tTA were most thoroughly evaluated in the thigh muscle, thymus, and tongue of the trans-

Table 1. Activation of the luciferase gene in tissues of single transgenic mice from line LU5 and in tissues from double-transgenic lines TA1/LU5, TA2/LU5, TA4/LU5, and TA5/LU5

Tissue	Luciferase activity, rlu/mg of total cellular protein (no. of animals analyzed)				
	LU5	TA1/LU5	TA2/LU5	TA4/LU5	TA5/LU5
Thigh					
− Tc	340 ± 160 (19)	10,400 ± 3,100 (4)**	14,800 ± 6,700 (7)*	32,300 ± 17,300 (9)*	53,300 ± 18,600 (4)*
+ Tc	390 ± 150 (7)	410 ± 290 (4)	90 ± 25 (7)	180 ± 110 (14)	1,500 ± 700 (4)
Post-Tc	ND	ND	ND	43,100 ± 6,000 (3)*	ND
Thymus					
− Tc	210 ± 140 (19)	6,000 ± 2,200 (4)**	11,400 ± 6,000 (7)*	5,300 ± 2,800 (9)*	1,800 ± 650 (4)*
+ Tc	230 ± 160 (7)	100 ± 30 (4)	170 ± 110 (7)	200 ± 140 (14)	290 ± 160 (4)
Post-Tc	ND	ND	ND	3,700 ± 1,000 (3)*	ND
Tongue					
− Tc	560 ± 210 (19)	26,000 ± 21,500 (4)**	12,900 ± 6,800 (7)*	27,200 ± 17,000 (9)**	45,700 ± 23,700 (4)*
+ Tc	520 ± 190 (7)	240 ± 140 (4)	180 ± 130 (7)	4,900 ± 3,800 (14)	1,300 ± 490 (4)
Post-Tc	ND	ND	ND	37,700 ± 8,100 (3)*	ND

Luciferase activities were measured in animals that had not been treated with tetracycline (−Tc), in those that had been treated with tetracycline pellets (+Tc), and in those whose tetracycline pellets had been removed 7 days earlier (post-Tc). ND, not determined. *, The difference of activity in the absence and presence of tetracycline (or in the absence of tetracycline and after removal of the tetracycline pellet) yielded a value of $P < 0.01$; **, the difference of activity in the absence and presence of tetracycline yielded a value of $P < 0.05$.

genic reporter line LU5. The basal luciferase activity in these tissues from 19 mice from line LU5 is reported in Table 1. In most mice, basal activity was close to background levels. On occasion, basal luciferase activities of up to several thousand rlu/mg of protein were measured in tongue and thymus. This suggests that there is some heterogeneity of transgene expression within a single integration site. In several mice, basal luciferase activity was analyzed in up to eight additional tissues (Table 2). The ability of the luciferase transgene in line LU5 to respond to activation by tTA was evaluated after breeding this line into the five trans-activator lines. No luciferase activity was measured in any tissue of mice transgenic for the LU5 and the TA3 locus (data not shown). This suggests that the line TA3 does not produce sufficient tTA to activate expression of the luciferase gene. The other four trans-activator lines produced sufficient tTA to activate the luciferase gene (Table 1). Trans-activation was observed in most tissues. The pattern of activity in the different tissues was similar to those reported for an hCMV *IE1*-CAT transgene (13) and an hCMV *IE1*-neo transgene (14). Levels of luciferase activity in the liver were low even in the presence of the tTA. This can be attributed to the low transcriptional activity of the hCMV *IE1* enhancer in liver cells of transgenic mice (13, 14).

Since endogenous DNA sequences in the vicinity of integration sites can exert strong position effects on the expression of transgenes, we compared the extent of induction by a single trans-activator line with three different luciferase reporter lines. Trans-activator line TA5 was bred into lines LU1, LU5, and LU6, and the magnitude of luciferase activity was measured. The tTA strongly activated the luciferase reporter gene in several tissues of all three lines (Table 2). The pattern of activation was similar in all three combinations. Specifically, expression in tongue, thigh muscle, and skin could be activated at least 100-fold. No activation of the luciferase gene was observed in the liver. The luciferase gene in an additional line did not respond to trans-activation (data not shown), suggesting that the reporter transgene was silent at this integration site.

The β-galactosidase reporter gene allowed us to analyze induction on a single-cell level. The four lines of mice carrying the β-galactosidase transgene were bred into trans-activator lines. No β-galactosidase activity was observed in mice from any of the four lines that contained only the β-galactosidase gene (data not shown). Blue-stained nuclei were observed in the thigh muscle, tongue, and seminal vesicles of mice carrying both a β-galactosidase reporter and a trans-activator gene from several combinations of adult double-transgenic mice. In all combinations tested, not all nuclei were blue, suggesting that only a subset of cells expressed both transgenes (Fig. 1). Blue-stained nuclei were less consistently found in the thymus, heart, kidney, and cerebrum from double-transgenic mice (data not shown). The tissues (tongue and thigh muscle) that demonstrated β-galactosidase activity in nearly all combinations tested were the same tissues that demonstrated high levels of luciferase activity. Activation of the β-galactosidase reporter gene by tTA was also analyzed during embryonic development. While blue staining was restricted to the spinal ganglia in day 11.5 postcoital embryos (data not shown), it was detectable in several embryonic tissues at postcoital day 16.5 (Fig. 2*B*). Particularly strong expression was seen in the nasal region, pituitary, choroid plexus, thymus, and pancreas of whole embryos. However, tissue sections revealed that not all nuclei were blue in these areas (Fig. 2*C* and data not shown). This nonuniform staining pattern was similar to that observed in sections from adult tissues (Fig. 1).

Luciferase Gene Activity Was Abrogated in the Presence of Tetracycline. To inhibit expression from the reporter genes, slow-release tetracycline pellets were implanted into mice transgenic for both the tTA gene and the luciferase reporter gene. Luciferase activities in thigh muscle, thymus, and tongue were measured after 1 week. Basal levels of luciferase activity were found in all double-transgenic mice receiving tetracycline (Table 1). This illustrates that tetracycline inactivated tTA in transgenic mice. Placebo pellets did not reduce luciferase gene activity (data not shown).

DISCUSSION

We have demonstrated that the tetracycline-responsive promoter $P_{hCMV*-1}$ has low basal activity in most tissues of transgenic mice. In double-transgenic mice that synthesize the tetracycline-responsive trans-activator tTA, $P_{hCMV*-1}$ was strongly activated in many tissues. The induction of gene expression was abrogated by the administration of standard therapeutic doses of tetracycline. No toxicity was observed from the exposure to tetracycline. This inducible promoter

Table 2. Activation of the luciferase target gene in transgenic lines LU1, LU5 and LU6, and induction by the tTA transcription factor from line TA5

Tissue	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction
	LU1	LU1/TA5		LU6	LU6/TA5		LU5	LU5/TA5	
Tongue	110	14,000	130	50	116,000	2300	590	45,700	80
Thigh	240	39,500	160	130	7,700	60	350	53,300	150
Liver	20	80	4	10	120	12	20	50	2
Thymus	40	1,400	35	10	8,100	800	220	1,800	8
Heart	50	400	8	110	1,600	15	20	12,300	600
Skin	110	6,700	60	9700	267,000	30	350	73,000	200
Duodenum	15	150	10	2800	21,000	7	10	1,200	120
Colon	80	800	10	170	9,000	55	100	600	6
Whole brain	700	750	1	20	1,600	80	70	500	7
Lung	100	30	0	20	1,500	75	300	300	1
Spleen	20	1	0	350	57,000	160	130	10,700	80
Kidney	10	170	17	10	2,900	290	50	400	8
Seminal vesicle	ND	ND	ND	80	404,000	5000	1000	1,323,000	1300
Testes	ND	ND	ND	1300	2,600	2	360	23,000	64
Uterus	1	470	470	ND	ND	ND	ND	300	ND
Ovary	1	90	90	ND	ND	ND	ND	1,900	ND
Mammary gland	50	1	0	ND	ND	ND	ND	ND	ND

ND, not determined.

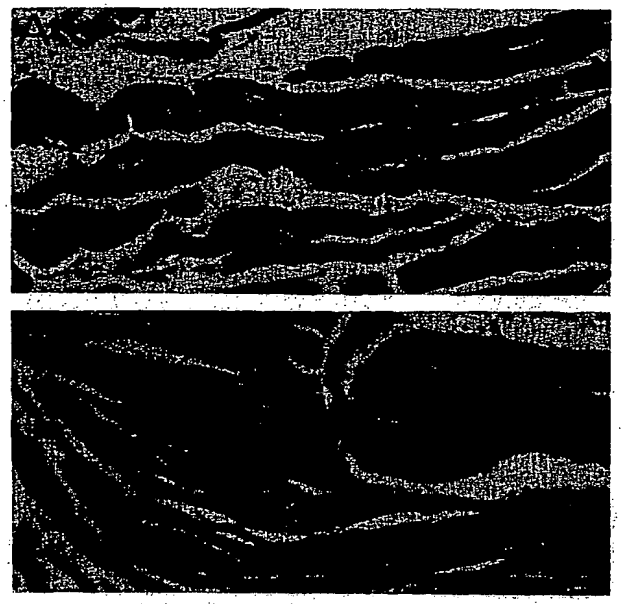

FIG. 1. β-Galactosidase activity in adult tissue sections from thigh muscle (*A*) and tongue (*B*) from an adult mouse carrying the G2 line β-galactosidase and TA1 transgenes. Cells with blue nuclei expressed the β-galactosidase gene.

system can therefore provide temporal control over gene expression in transgenic animals. It has been shown recently that the same system is functional in transgenic tobacco plants (15).

Basal Gene Activity. An essential feature of any inducible system is low promoter activity in the inactive state. Basal transcriptional activity in the *tet* repressor/VP16 system is dependent upon the promoter elements of the target gene. The activity of the $P_{hCMV*-1}$ was examined by using both the luciferase and β-galactosidase reporter genes. No measurable β-galactosidase activity was seen in mice carrying only the β-galactosidase transgene. However, measurable levels of luciferase activity were occasionally observed in certain tissues from some mice containing only the luciferase transgene. The fact that basal expression from the β-galactosidase gene was not seen is probably due to the greater sensitivity of the luciferase assay. The sporadic occurrence of measurable levels of luciferase in some mice indicates that there can be transcriptional activity from the hCMV *IE1* core promoter (16, 17). A core promoter containing only a TATA box may provide lower baseline activity (18). However, this suggestion will have to be tested to determine if such a skeletal promoter can be activated when embedded in chromatin.

Inducibility and Repression of Transgenes. Binding of the tTA transcription factor to the *tet* operator sequences in the promoter of the target gene activates transcription. High activation levels can be achieved even at low concentrations of tTA (9). The fact that tTA is capable of activating transcription of target genes in several independent chromosomal loci shows that the trans-activator can gain access to bacterial control elements packaged into chromatin in differentiated tissues. Trans-activator protein produced by four independent lines of transgenic animals activated luciferase target genes to a similar degree. This may indicate that position effects do not have a dramatic influence on the magnitude of target gene activation. Activity of the luciferase gene in double-transgenic mice was abrogated by administering tetracycline. The therapeutic levels of tetracycline released from the subcutaneously placed pellets were enough to interfere with binding of tTA to the *tet* operator sequences of $P_{hCMV*-1}$. The effect was reversible after removal of the tetracycline pellet. Specific evaluation of the kinetics of repression of gene expression following tetracycline administration or release of repression after tetracycline withdrawal was not made in this study. However, we can state that luciferase activity in double-transgenic mice was fully repressed after 7 days of tetracycline administration. Induction of gene expression followed withdrawal of tetracycline.

Variability and Mosaicism. Expression of the luciferase gene in mice carrying also the hCMV-tTA gene varied between animals from any given line and even between littermates. This variability may be inherent in the hCMV *IE1*

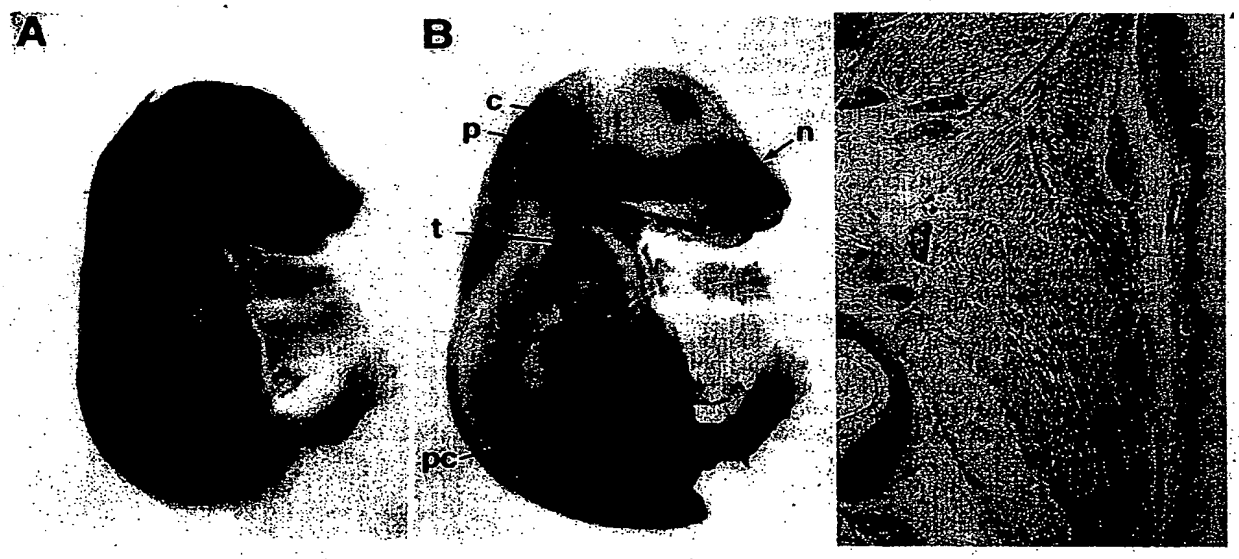

FIG. 2. β-Galactosidase activity in bisectioned day 16.5 postcoital embryos. (*A*) Embryo carrying the G4 line β-galactosidase transgene. (*B*) Embryo carrying the G4 and TA4 transgenes. c, Chloroid plexus; n, nasal region; p, pituitary; pc, pancreas; t, thymus. The blue staining of intestinal tissue is due to endogenous β-galactosidase activity. (*C*) Section from the neck region of the embryo shown in *B*. Cells with blue nuclei expressed the β-galactosidase gene.

promoter/enhancer used to direct expression of the trans-activator. Such variability was also observed in littermates carrying a hCMV *IE1*-CAT transgene (13). Histological analyses for β-galactosidase activity revealed mosaicism. The number of cells expressing the transgene was only a subset of the cells expected to stain in that site, a pattern reminiscent of position-effect variegation (19). Although a possibility, mosaicism cannot be attributed simply to the use of the β-galactosidase reporter gene as this gene has been expressed homogeneously in embryonic, fetal, and adult tissues (20). Mosaicism has been observed with other transgenes (12, 14), and even some endogenous genes (21), and is referred to as incomplete penetrance (12). This stochastic pattern of gene expression may reflect the activity of certain endogenous genes (21) and also may be the cause of the variable penetrance of defects observed in null mutant mice (22, 23).

Other Inducible Systems. Other inducible promoter systems have not offered the degree of control presented by this strategy. Similar to the system reported here, two previously described binary systems consist of a silent target gene that is induced by constitutive or regulated trans-activators (2, 3). In the third reported system, a silent target gene is activated by a site-specific recombinase (4). However, in contrast to the system described here, transgene activity in these binary systems is regulated by the transcription pattern inherent to the promoter controlling the trans-activator gene. No additional manipulation is possible.

A system that uses the *tet* repressor to inhibit gene transcription is another approach to controlling gene activity. In these systems *tet* repressor molecules bind to *tet* operator sequences located at the transcriptional start site and block gene transcription in the absence of tetracycline. In the presence of tetracycline binding of the tet repressor to the *tet* operator is greatly reduced and transcription is activated (24). Repression of transcriptional activity to basal levels has been achieved in the presence of ≈500,000 tet repressor molecules per cell. Such a concentration can only be obtained with strong promoters, such as the 35S promoter from the cauliflower mosaic virus (24). Therefore, it may be difficult to achieve a repression of the transgene with housekeeping or standard tissue-specific promoters.

The induction of transgenes through the withdrawal of tetracycline can have specific advantages for some experiments. For example, when analyzing the roles of oncogenes, growth factors, or tumor suppressor genes on tumor formation, a long period of gene activation may be required (25–28). If a tetracycline-responsive promoter is used to control oncogene expression, it may be convenient to have the animals off tetracycline during this time.

In conclusion, the tetracycline regulatory system can provide temporal control of transgene expression. It should be useful for experiments designed to address certain biological questions in transgenic animals. For example, temporal control of the induction of growth modulators, oncoproteins, and other proteins participating in developmental processes could provide further definition to their roles in normal growth and tumorigenesis. The effects of expressing potentially deleterious genes can be studied, since these genes can be rendered inactive by using tetracycline. Alternatively, the system can be combined with one of the site-specific recombinases (4) and used to delete genes at specific time points during development.

We thank Kirsten Backs and Uli Francke for generating transgenic mice, Rainer Libal for maintaining the mouse colony, and Jia Di Hu, Eva Kubiczek, and Minglin Li for technical assistance. L.S.O. was supported by a European Molecular Biology Organization fellowship. P.A.F. was on a leave of absence from the University of Maryland Medical School, and L.H. was on a sabbatical leave from the National Institutes of Health. P.A.F. and L.H. are grateful to the Alexander von Humboldt Stiftung for generous support. This work was in part supported by the Deutsche Forschungsgemeinschaft SFB 229.

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